COMPARISON OF THE ASYMMETRY OF THE HEXOSE TRANSFER SYSTEM IN THE RED CELL OF THE NEWBORN GUINEA-PIG AND HUMAN BY PERMEABILITY STUDIES

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by

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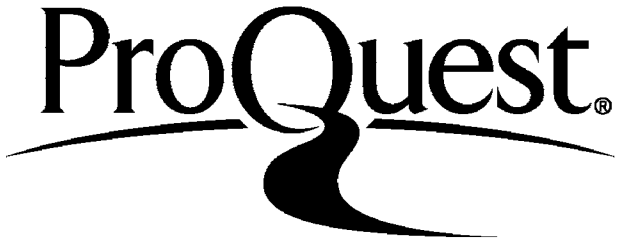

Abstract

A comparison has been made between the hexose transferring system of erythrocytes from fetal and newborn guinea-pig cells and that of the human erythrocyte.

The kinetic parameters of 3-O-methyl glucose exchange in fetal and newborn guinea-pig cells have a higher constant for maximal exchange but show a lower affinity than in human erythrocytes.

Both systems show asymmetry of affinities toward non-transportable inhibitors such as 4-6-O-ethylidene-α-D-glucopyranose (ethylidene glucose) and methyl-2,3-di-O-methyl - α-D-glucopyranoside (trimethyl glucoside) but that in newborn guinea-pig cells is less than that in human erythrocytes.

Cytochalasin B competitively inhibits hexose exchange in newborn guinea-pig cells as it does in human cells but the K_i value was larger suggesting a lower affinity. Exits were inhibited in an apparently non-competitive manner as occurs in human cells.

Cytochalasin B binding was studied and indicated that there were fewer hexose transferring components in newborn guinea-pig cells but as the maximal value for exchange is greater it may be presumed that the turnover number must be larger.

The inhibition of the two systems by the biphenolic inhibitors phlorizin, phloretin and polyphloretin phosphate was similar but there were differences in the K_i values.

The sensitivity to the irreversible inhibitors fluoro-dinitrobenzene and bromo-dinitrobenzene were also similar but there were differences in the effects of incubation in the presence of the transportable sugar 2-deoxyglucose.

It is concluded that the hexose transferring system in the two species is very closely related and that the differences are not more than might be expected in functional proteins taken from divergent species.

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CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

1. General
2. The erythrocyte
3. Fetal red blood cells
4. The red cell membrane
5. The hexose transfer system in the human erythrocyte
6. The hexose transfer system in erythrocytes from other species
7. Postnatal changes
8. Inhibitor studies
9. Present problem

General

The study of transport through biological membranes is an immensely vast subject. The investigations into the transport processes have involved the use of an equally vast range of membrane sources. Animal, plant and bacterial cells have been the primary sources, however more recently artificial membranes have been introduced into this field.

In the middle of the 19th Century, Schwann and Schleider suggested that the unit of behaviour in living organisms was the cell. As may be obvious, this proposition has held good to the present day.

The internal environment of all cells is very different from their surroundings, however, there is a constant exchange of metabolites, waste products and other substances between these two environments. It is this constant exchange of materials that determines the activity, or the physiological state of the cell. The concept of permeability or rather semi-permeability has had to be postulated to account for a vast bank of data accumulated over a long period of time and from the observations on the behaviour of the cell under various conditions. These observations range from the differences in the internal and external environments, both in terms of presence and concentrations of electrolytes and non-electrolytes, to the demonstration of the presence of free electrolytes in the interior of the living cells. The concept of cell membranes and their properties have been extensively reviewed by Ponder (1966).

As is so often the case, with many biological systems, the advances lag behind and are often dependent upon developments in other fields. This for example is evident with various technical developments in instrumentations, thus as techniques, such as X-ray diffraction, calorimetry, nuclear magnetic resonance and various forms of optical spectroscopy have developed, the concept of the plasma membrane, being a thin layer of material that separates the inside of the cell functionally from its outside medium, has gathered great momentum. (See for example, Introduction by Gomperts, 1977). Additional and supportive description of the membrane structure has been obtained from studies with the electron microscope. (See Review by Robertson, J.W., 1964).

The development of a selective permeability barrier must have been of the greatest importance to the development of the primitive cells. These cells presumably arose from the formation of membranes around a few macromolecules, possibly possessing catalytic properties, thus allowing smaller molecules and ions to interact. These selectively permeable barriers would hence permit selection and retention of essential molecules and at the same time allow exclusion and excretion of other molecules. (Goldacre, 1958).

The permeability of cell membranes differs widely for different substances. The smaller molecules move more freely through the membranes than do larger molecules with similar chemistry and non-electrolytes move more freely than electrolytes. This selectivity of membranes

would thus prevent the uncontrolled diffusion of substances into and out of cells, hence allowing for a greater control and order in its biochemical processes for physiological functions. In many cases the cells do not wait passively for these boundary interchanges to occur, but intervene actively, with the expenditure of energy, in the process. The subject of water and ionic permeabilities across the red blood cell membranes has been reviewed by Passow (1964) and the permeability to sugars will be discussed later in the chapter.

The membrane has thus far evolved to the extent that there are a range of transport processes working simultaneously and in unison to maintain the integrity of the living cell (Stein, 1967). Briefly, amongst these transport processes the simplest form is that described as passive transport or passive diffusion. This is a situation where the movement of substances is in the direction of a physical gradient, for example, concentration, potential, hydrostatic pressure or osmotic pressure. Another much more specific process, the active transport process, is that where the movement of a substance across a membrane is irrespective of the concentration gradient. Such movement requires the active expenditure of chemical energy to establish or maintain a gradient. Yet another process which is highly specific and where the movement of substances across the membrane is via specific interaction with a limited number of loci on the membrane is now well recognised. This process which occurs without the expenditure of metabolic energy is several orders of

magnitude faster than passive diffusion. This is termed facilitated diffusion, or carrier-mediation. (Danielli, 1954; Widdas, 1954).

The Erythrocyte

In the embryo, erythopoiesis begins in all mammals shortly after the formation of the germ layer (Bloom, 1938). It starts in the wall of the yolk sac where mesenchymal cells retract their processes to assume a more rounded form, free from other cells. All types of blood cells arise from these hemoblasts. A similar transformation is encountered later in the body mesenchyme, liver, spleen and bone marrow, as these structures develop. It is only at a later embryonic stage that there is a division in the blood forming tissues into myeloid tissue for elaboration of red corpuscles, granulocytes and lymphatic tissue for the production of lymphocytes. Possessing neither the nucleus nor any mitochondria, the red cells are the final product of hemopoiesis.

The red cell is one of the commonest cells to be found in the animal kingdom, and has been used for a systematic study of permeability for a range of substances. It is noted that the red cell is still a favourite for the study of transport processes across membranes. Its relatively simple structure allows a more detailed and varied approach than is possible with more complex cells. Although relatively simple, these cells show common fundamental features of regulating the entry and exit of substances.

The features that make the erythrocyte a very desirable cell to use for the study of biological transport systems are many fold. The choice of erythrocytes for any study has been primarily dictated by its availability with an absolute minimum of technical and/or surgical complication, also the ease with which these cells may be handled in terms of washing and separation from their bathing medium is an advantage. Red cells have been used in spite of their population not being homogeneous.

As described below, other properties which have made the erythrocyte a favourite choice amongst workers in the field of sugar transport across membranes, range from its appearance as a pigmented cell with high refractive index to its structure which allows changes in volume without effective changes in the area of its membrane.

Firstly, the erythrocyte with a high concentration of hemoglobin enables the cell to be used for photoelectric measurements. The Sen-Widdas technique (1962a, b), using the Ørskov (1935) type of apparatus (Widdas, 1953), depends on this and the unique property of the erythrocyte to act as a perfect osmometer. This procedure (discussed later in greater detail) is a relatively fast and straight-forward method for determining kinetic parameters of the hexose transfer process.

Secondly, as mentioned above, the erythrocyte is devoid of the nucleus, mitochondria and all other subcellular structures, thus eliminating any complications that may arise due to compartmentalization.

Thirdly, the plasma membrane of the erythrocyte has been studied most widely, in terms of chemical composition and molecular structure (Guidotti, 1972; Steck et al., 1971), thus making available a whole library of information for the researcher.

Fourthly, when exposed to hypotonic solution, which causes osmotic swelling, the erythrocyte membrane is disrupted, allowing large molecules to enter or leave the cell. On restoration of isotonicity, the cell membrane regains its original permeability characteristics, thus the cell may be 'loaded' to any desired composition. As may be appreciated, this is potentially a very powerful technique which may be used in studying membrane based activities in the absence of all or most of the cytoplasmic contents, and also in studying the activities with predetermined internal and external environments. (Gardos , 1954; Hoffman, 1958; LeFevre, 1961).

The large number of cells that are present in every cubic millimetre of blood, ensures that the mean behaviour of a large population of cells is observed.

The carrier-mediated transport mechanisms which are present in erythrocytes are now considered to be an essential feature of biological transport processes and hence this may be another reason why the erythrocyte is favoured for study. (Stein, 1967).

Fetal Red Blood Cells

It is well known that the red blood cells from fetal or newborn animals are quite different from those from a normal adult of the same species. There are differences in their number, size, mean corpuscular volume, and oxygen transport capacity (Windle, 1941).

In comparing red cells from various species, Wintrobe and Shumaker (1936) concluded that the red cell counts, amounts of hemoglobin and the volume of packed cells was low in very young fetuses as compared with adults of the same species. The red cells were very much larger than those of the adult and a greater number were immature, as judged by the presence of a nucleus or supravitally stained reticulum. As the age of the fetus increases the red cell count, amount of hemoglobin and volume of packed red cells tends to rise, whereas the mean size of the erythrocyte decreases. They also concluded that the longer the gestation period the more nearly do the red cells at birth correspond to those of the adult and vice-versa.

In as far as true differences are concerned, the hemoglobin in the fetal red cells has been shown to be different from that in the adult (McCarthy, 1933). The difference in the avidity for oxygen is inexplainable solely on the basis of different pH of the maternal and the fetal bloods, but is a consequence of actual physical and chemical differences between the two hemoglobins (Windle, 1941).

There are also differences in permeability characteristics between the fetal and newborn and the adult red blood cells. The human fetal and newborn red cells have a lower permeability to water than do adult human red cells (Sjolin, 1954). This is also true for the sheep (Widdas, 1951). The ionic distribution, indicates that the adult red blood cells may have a faster leak relative to 'pump' for potassium than the fetal and newborn cells, which contain a higher concentration of potassium. (Wise et al., 1947; Widdas, 1954). The fetal and newborn red cells in most mammals are permeable to glycerol, erythritol (Bowyer and Widdas, 1955), and vitamin C (Räihä, 1958), however, the permeability for these substances is often diminished or lost in the adult cells.

The differences or similarities between fetal bloods, in terms of the hexose transfer system present in the erythrocyte membrane is discussed later in this chapter.

The Red Cell Membrane

In any study of transport of substances across a membrane, it is essential first if possible to define as many parameters as possible. To be able to fully appreciate the implications of experimental observations, the membrane structure shall be outlined briefly.

Essentially, the red cell membrane is composed of three classes of components, namely proteins, lipids and saccharides. These membrane components are held together in their particular orientations and conformations by

Table Ii

Composition of red cell membrane (after Rosenberg and Guidotti, 1968).

Component	%
Protein	49.2
Lipids (Total)	43.6
Phospholipids	32.5
Cholesterol	11.1
Carbohydrates (Total)	7.2
Neutral Sugar	4.0
Hexosamines	2.0
Sialic acids	1.2

non-covalent interactions, such as hydrophobic and hydrophilic interactions, hydrogen bonding and electrostatic interactions. Of the membrane components, the lipids are the main structural material, in the form of a bilayer. A major fraction of around 30% of the total lipid content is cholesterol. The remaining fraction is made up of various phospholipids, such as phosphatidyl choline, phosphatidylethanolamine, sphingomyelin and phosphatidylserine (Van Deenen and De Gier, 1964). A summary of the constituents of red cell membranes is given in Table Ii.

The proteins, which constitute a large proportion of the cell membrane material are of two classes. First of these, the peripheral proteins are those that are loosely bound to the membrane skeleton, hence their dissociation may be achieved with mild treatment. Thus only the very weak electrostatic interactions are proposed to be responsible for binding these proteins to the membrane. Secondly and more importantly, the integral proteins, are bound firmly into the lipid-bilayer framework, hence are characterised by their reluctance to be isolated from the bulk of the membrane material. These proteins are responsible for various enzymatic and transport activities (Singer and Nicolson, 1972).

Some of the saccharides that are found in the membranes are in association with proteins and lipids and are in the form of glycoproteins and glycolipids. Other saccharides appear on the surface of the membranes and may have an immunological significance.

After thermodynamic considerations and experimental observations, the membrane is pictured as a mosaic structure, in which globular proteins alternate with sections of the phospholipid bilayer in the cross-section of the membrane (Singer and Nicolson, 1972).

The Hexose Transfer System in the Human Erythrocyte

The transport of glucose across membranes, from various tissues, has been extensively studied during the last few decades. These investigations have involved the use of erythrocytes, adipose tissue cells, ascites-tumour cells, bacteria and yeast, brain-slices, muscle, renal tubules, mammalian intestine, rat diaphragm, perfused heart and the placenta. Although a sugar transfer system is present in all the examples listed above, the actual mechanism in different tissues is different.

The hexose transport process across the human erythrocyte is very well documented (Miller, 1969; Jung, 1975; LeFevre, 1975a; Naftalin and Holman, 1977). It may however, be of interest to mention briefly the other processes at work first.

The hexose transfer system in muscle has been reviewed by Stadie (1954) and that in adipose cells has been described by Vinten et al. (1976) and Whiteshell and Gliemann (1979). The hexose transfer system encountered in cells from these two tissues differs from that in the erythrocytes only in its response to insulin (Levine et al., 1949; Park et al., 1956, 1968; Helmreich and Cori, 1957;

Kipnis and Cori, 1957). There is an increase in the penetration rate into these cells with insulin, however the penetration of hexose into the erythrocyte is unaffected by insulin (Wilbrandt et al., 1947).

The uptake of sugar in bacterial and yeast cells is of yet another class (Wilbrandt and Rosenberg, 1961). The transport mechanism in these cells is able to transfer sugar against a concentration gradient. This is essentially an energy-coupled carrier-mediated system, the so-called group translocation mechanism (Kaback, 1970; Lin, 1971; Oxender, 1972; Kotyk and Höfer, 1965). The transfer system across the intestine, although utilizing metabolic energy, is distinctly different from the group-translocation process. The transport of sugars across the intestine involves a coupling between the sugar and the sodium ion transport processes. The transport of one being dependent upon the presence of the other and vice-versa.

Finally, the transfer system that is present in the erythrocyte membrane is one of the most studied of all the sugar transport systems. As the present study has also been concerned with the investigation of this system, it shall be briefly discussed here.

The carrier mediated hexose transfer across the human erythrocyte has been primarily based on kinetic observations on the movement of glucose across the red blood cell and the placenta. The existence of the system was formalised after confirmation of several anomalies associated with the movement of glucose across these

membranes. The first of these anomalies was the deviation from Fick's law at high sugar concentrations (LeFevre, 1948; Widdas, 1952). The rate of penetration of glucose was found not to be proportional to its concentration gradient. Another anomaly encountered was that which contradicted the Overton theory of permeability depending on lipid solubility. Rates of penetration of sugars were not proportional to their lipid solubility as was shown by Kozawa (1914), who investigated the permeability of erythrocytes to various mono- and disaccharides and found the cells to be impermeable to disaccharides. The above observations ruled out the possibility of passive diffusion being responsible for sugar movement across the erythrocyte membrane. Furthermore, various physicochemical studies showed that the process was of a very specific nature and that the chemical structure of the substrate determined the transport rate. Inhibition of the sugar transfer by various drugs and chemicals suggested the involvement of a specific membrane component (LeFevre and LeFevre, 1952). It was the realization of these anomalies that led initially to the formalization of the carrier-mediated transfer system in its simplest forms.

The carrier-mediated transfer of sugars is therefore characterised primarily by its speed of movement, saturation and competition kinetics, specificity and inhibition by chemicals.

The most remarkable feature of the carrier-mediation is its high unidirectional flux. The rate of utilization of glucose is around 100 times greater than non-specific supply of this sugar i.e. by simple diffusion (Jung, 1974). The human erythrocyte hexose transfer system is however capable of supplying glucose roughly 250 times the rate that is required for utilization (Widdas, 1954). At high concentrations of the substrate the carrier system, due to the limited quantity of the membrane component, is saturable and the flux is not proportional to the sugar concentration gradient but approaches a limiting value (LeFevre and LeFevre, 1952). This is a direct departure from Fick's law for simple diffusion. The saturation kinetics are, however, in no way solely indicative of the involvement of a true carrier. Note should be made of the fact that saturation kinetics can also be derived if one considers diffusion via passageways with a cross-sectional dimensions not substantially larger than the diffusing particles. This is particularly evident if only a single-file migration is allowed. Zierler (1961) demonstrated this phenomena using a gross mechnical model. Saturation in the erythrocytes is determined by a carrier-substrate affinity factor, i.e. various hexoses saturate the system at different concentrations (LeFevre, 1962).

As a consequence of a limited number of hexose transport 'carriers', mutual inhibition is exerted if two or more hexoses are present. The saturation phenomena is really a special case of this, where molecules of the same species

are competing for the same site of transfer. A prediction that all hexoses utilizing this system would have the same maximal transfer rate has been supported experimentally (LeFevre, 1962).

The hexose transport system across the erythrocyte membrane is highly specific. Its specificity extends to enantiomorphs. D-xylose is readily transported while L-xylose is virtually excluded (Wilbrandt, 1947). LeFevre in 1961 & 1962 investigated specificity sequences for most hexoses and pentoses quantitatively and expressed these in terms of substrate-carrier affinity. He showed that the affinity varied over three orders of magnitude, while the maximal transfer rate appeared virtually identical for all the sugars. The simplest interpretation of specificity of this nature is that the substrate must reversibly combine with a fairly complex membrane component involved in the transfer process, for its translocation.

The structural requirements of this system have also been investigated by LeFevre (1961, 1962, 1972) and LeFevre and Marshall (1958). Generally the aldose is preferred to a ketose, the D-isomers are preferred to L-isomers, five and six carbon, linear or cyclic polyhydric alcohols are totally rejected. An interesting correlation has been observed between the affinity of a sugar to the carrier and the stability of this sugar in a particular, $^4C_1$ chair conformation. The more stable the sugar is in this conformation, the higher is the affinity of the carrier for this sugar (LeFevre and Marshal, 1958; LeFevre, 1961).

Barnett et al. (1973) using substituted D-glucose and D-galactose derivatives revealed the relationship between the hydrogen bonding with the carrier and the spatial requirements thereof. Essentially, they found that no single hydroxyl group was vital for stereospecific requirements and that the hydroxyl gorups at C-1, C-3 and C-6 position played an important role in the association of the sugar with the carrier molecule. Furthermore, the hydrogen bond at the hydroxyl group on the C-1 position was only effective when it was present in the β-D-gluco configuration. Also the involvement of the C-4 hydroxyl group in hydrogen bonding was indicated. The hydroxyl group at the C-2 position appears to have no hydrogen-bonding activity at all, since with 2-deoxy-D-glucose the affinity for the carrier is slightly higher than with D-glucose. An altogether different process of interaction may be involved at C-6 of the D-glucose. It is thought that the carrier region around C-6 may contain both the hydrophobic and the polar binding groups. In conclusion therefore one may envisage the carrier to form a pocket around C-1 and C-3 and at the same time allow C-4 and C-6 to remain exposed to the surrounding medium (Barnett et al., 1973).

Besides mutual inhibition exerted by sugars, the transport system shows sensitivity to various classes of drugs. The mode of action of these drugs can be classified in classic enzymatic terms. That is to say in terms of type of reaction, namely competitive, non-competitive reversible and

non-competitive irreversible. The underlying inhibitory mechanism of each drug used in the present study and implications thereof have been discussed in the appropriate chapters.

Attempts to develop models to describe the behaviour of sugar passage across membranes have been undertaken from as early as 1948. LeFevre (1948), LeFevre and LeFevre (1952) described a model whereby the sugar complexed with an enzyme-like component in the membrane, possibly a phosphorylating enzyme. The membrane component once complexed with the substrate was seen to catalyse a change in the position of the substrate relative to the diffusion barrier. For this mode, they assumed that the breakdown of the complex was rate limiting. This model however proved unsatisfactory in explaining saturation.

Widdas (1952) proposed a similar model for the transfer of glucose across the placenta. The feature of this model which was used to derive the kinetics was the ability of the membrane component, the carrier, to move across the membrane and thus in doing so, carry the sugar across. This model proposed the rate limiting step to be the slow movement of the carrier across the membrane, rather than the dissociation of the sugar-carrier complex. Other simplifying assumptions proposed in the simpler forms of this model are the symmetry of sugar-carrier affinities at the two sides of the membrane and also the symmetry between the rates of transfer of the free and the complexed

Table Iii

The different half-saturation constants, for glucose and galactose, obtained using different experimental procedures, at 20 °C.

Procedure (Efflux measurements)	Sugar	Half-saturation Constant (mM)	Reference
Zero-trans	glucose	25	Karlish et al. (1972)
	galactose	165	Lieb and Stein (1972a)
Infinite-cis	glucose	1.8	Miller (1968b)
	galactose	12	Miller (1965)
Equilibrium Exchange	glucose	38	Miller (1968b)
	galactose	147	Lieb and Stein (1972a)

(Modified from Table in the review by Jung, 1975)

carrier between the two membrane faces. This model proved to provide a consistant framework for the interpretation of a wide variety of kinetic data for the movement of sugars across the red cell membranes (LeFevre, 1975b).

The difficulties with this model only became apparent after the introduction of new techniques for observing the passage of sugars across membranes. (Miller, 1968a, b). The main discrepancy between the theoretical Widdas' model and the experimental observations was the difference in the affinity constant, which varied widely when different experimental procedures were used in its determination (see Table Iii). Another anomaly encountered by Miller (1968a, b; 1969) was the inconsistency in the quantitative prediction of the counter-transport time course for glucose. Counter-transport is defined as the movement of a sugar under the influence of competition with another sugar, which is asymmetrically distributed across the cell. This results in one sugar driving the other against its concentration gradient. The counter transport of labelled mannose or galactose into cells heavily preloaded with the unlabelled form of the sugar agreed very well with theoretical predictions, however, similar experiments with glucose did not seem to conform to prediction. The radioactive glucose accumulation peaked far too rapidly and also peaked to too low a level. It was also observed that the presence of an appreciable concentration of a sugar on the 'trans' side stimulated the transfer of the

'cis' sugar. This, the 'trans-stimulation', phenomena is also contrary to the Widdas (1952) model, which may however be reconciled with it if one assumes the mobility of the loaded carrier to be greater than that of the unloaded carrier. (Levine et al., 1965; Mawe and Hempling, 1965).

Yet another anomaly that may be reconciled with different mobilities of loaded carrier, is one that was also observed by Miller. Miller (1969) demonstrated that D-glucose efflux was much greater if the 'trans' sugar was either D-mannose or D-galactose than if it was D-glucose itself. This apparent anomaly may be partly accounted for in terms of competition of the trans sugar that had entered the cell during the period of measuremnt (Levine, Oxender and Stein, 1965).

The appearance of these anomalies led to considerations of new models together with attempts to reconcile the mobile-carrier theory with the newer experimental data.

There are primarily three mechanisms put forward in all the new models. The first being the complete abandonment of the mobile-carrier concept. These transport models involve 'fixed-sites' or non-mobile carriers, and the transfer of the sugar is proposed to occur via these fixed loci. The second concept involves a resistance in the form of an unstirred layer adjacent to the membrane, through which sugar must first diffuse. The second concept has the above criterion as an addition to the simple mobile-carrier system. Finally the third

type of mechanism abandons only the simplifying assumptions of the original Widdas model. Thus the latter mechanism takes into account various rate-limiting steps, alteration in the rate of transfer of the complexed carrier and the asymmetry of affinities between the interior and the exterior sites, together with asymmetry of movement of the carrier in the two directions.

Keeping the limits of this study in mind, it is also of interest to briefly outline some of the alternative kinetic models.

Of the non-mobile type of carrier mediated transfer system, Lieb and Stein (1970, 1971) described a complex model which was called the 'Internal transfer Model'. This model envisaged the transport occurring by the internal transfer of sugar via a tetrameric protein, composed of two pairs of sub-units. Each sub-unit in turn, having one high affinity binding site and a low affinity site. The tetramer encloses an internal cavity and the whole unit can exist in either of two conformations. The conformational change which is associated with the trans-location of the sugar between the outside medium and the internal cavity is substrate induced. Thus the sugar may bind to the carrier at either side of the membrane and also exchange of two sugar molecules may occur within the internal cavity.

This model accounts well for the primary kinetic data concerning differences between net and exchange fluxes. This model, although much more complex than the simple mobile-carrier model, does not account for anomalies reported by Miller (1968a) on counterflow, nor does it provide an interpretation for the initial rates of Hetero-exchange. Hetero-exchange is the exchange of one species of sugar with another. The first of these discrepancies has however, been discounted on technical grounds (Lieb and Stein, 1970) and the second on the basis of analysis by Eilam and Stein (1972).

In the same year, another non-mobile carrier model, the 'fixed lattice' pore model was proposed by Naftalin (1970). This was a modification of an earlier single-file 'pore creep' model of Zieler (1961). This particular model considered a polar pore spanning the membrane containing a number of fixed sugar binding sites. The pore was proposed to be large enough to allow sugar molecules to pass each other, in opposite directions. Naftalin's model also features a substrate facilitation property, whereby the exchange of sugar molecules between two adjacent sites occurs more readily than simple migration to an unoccupied adjacent site.

This model describes most of the basic features of the facilitated transfer system. It however lacks in explaining the trans-stimulation phenomena and also the difference in affinity constants as measured by different procedures. These inconsistances made it necessary to postulate a

significant unstirred-layer on the outside of the cells. The existence of this unstirred-layer has been challenged by Lieb and Stein (1972b) and Miller (1972). A recent critical experiment demonstrating the unimportance of the unstirred-layer effect is that with chlorpromazine. It was found that chlorpromazine inhibits net efflux of glucose whilst the exchange flux remains unaffected. (Baker and Rodgers, 1973). Another experiment (Zipper and Mawe, 1972) with insulin also demonstrated this unimportance. They found insulin increases net efflux without affecting exchange flux of glucose. Thus both these experiments reject the hypothesis that an unstirred-layer determines the trans-acceleration phenomena.

The substrate conditioned 'introversion' model is yet another modification of the non-mobile carrier mediation concept (LeFevre, 1973). This model employs a two column rather than Naftalin's (1970) multi-column matrix. The introversion model is seen to possess fixed sites in each of the layers, in a bilayer membrane. These fixed sites can exist in either of two conformations. The extraverted state which allows interaction with the adjacent aqueous medium and the introverted state which allows interplay with sites of opposite layers. The sites in these two states are in an equilibrium determined by occupancy of the sites by a suitable substrate. The process can work both unidirectionally or bi-directionally.

A unique feature of this transfer mechanism is that the affinity difference between various sugars is not at the

complexing site, rather it is at the conformational change, which is associated with the translocation process.

This model predicts, that at low D-glucose concentrations, the rate of L-glucose should be accelerated. This stimulation of L-glucose flux is not detected at any concentrations of D-glucose. This model is also inadequate in explaining why the maximal transfer rates for entry of sugar into sugar-free cells should be less than the maximal transfer rate for exit of sugar from cells suspended in sugar-free medium, particularly when the model proposes symmetry between the two sides of the membrane.

A more recent hexose transfer model that has been formulated by Holman (1980) also postulates fixed pores spanning the width of the cell membrane. Holman's (1980) allosteric pore model is based on both the concept of a multi-unit protein 'carrier' (Lieb and Stein, 1970) and the concept of a fixed lattice pore (Naftalin, 1970). The model envisages transport via a protein molecule with three functional subunits, only two of which are accessible for binding. The substrate enters the pore from either side of the membrane and the transport pore is allowed to assume one of several substrate-induced conformational states, which are determined by whether the pore is singly or doubly occupied and whether the occupancy is from the inside or the outside or indeed from both sides simultaneously.

Thus the allosteric pore model (Holman, 1980) explains the differences in the K_m and V_{max} values for hexose transfer as measured by different experimental procedures on the basis of co-operativity which is similar to that observed with typical allosteric enzymes.

In conclusion, therefore, the concepts of non-mobile reacting sites in the membrane have solved some anomalies but have not given a completely satisfactory explanation for all types of kinetic data.

The earliest attempts to reconcile the classical mobile-carrier transfer mechanism were to relax some of the restrictions on relative rate constants.

Regen and Morgan (1964) proposed that some of the over-simplifying assumptions in the classic model may be a reason why there were deviations between prediction of the kinetic model and the observed experimental findings. They showed that the essential features of the transfer system could also be derived from an asymmetrical arrangement. The only constraint being, that the relative magnitude of the rate constants were such that the system obeyed the second Law of Thermodynamics.

The kinetics of an asymmetrical transport process were described by Geck (1971) to account for the difference between the K_m and V_{max} for net-exits and net-entry and also for equilibrium exchange of sugars. This model also explained, to some extent, Millers' (1968a) hetero-trans stimulation and counterflow anomalies.

The concept of asymmetry has gathered momentum following an extensive accumulation of information on various aspects of the hexose transfer system in the erythrocyte. Historically, asymmetry was first suggested by Wilbrandt (1954), from his findings, that the phloretin group of compounds inhibit glucose exits much more than glucose entry. However, it was shown that the results were consistent with there being different degrees of competition exerted by the inhibitor in the different experimental procedures (Bowyer and Widdas, 1958). Bowyer and Widdas (1958) produced similar evidence for asymmetry, with the finding that inhibition of exits developed faster than inhibition of entry with 2,4-Dinitroflurobenzene (DNFB). The DNFB effect could not be dismissed with the interpretation as for the phloretin groups of inhibitors, since DNFB is a non-competitive irreversible inhibitor. That the non-competitive inhibitors P-chloromercuribenzene sulphonate, 3,3-di-2-chloroallyldiethylstilbestrol and N-ethylmaleimide also inhibit exits of glucose more than its entry into the erythrocyte was shown by Batt and Schachter (1973).

More direct evidence of this asymmetry in the transfer system in the human erythrocytes was put forward by Baker and Widdas (1972, 1973a) using non-transportable inhibitors. They showed that 4,6-O-ethylidene-D-glucopyranose (ethylidene glucose) is a potent inhibitor of the hexose transfer system as measured by the inhibition of glucose exits. They also showed that the ethylidene

glucose penetrated the cells by a process of simple diffusion and that it was not capable of inducing counterflow. This inhibitor, they showed, inhibits the transfer system asymmetrically. That is to say, the inhibition produced by 200 mM ethylidene glucose when present only on the inside of the cells was no more than that produced by 25 mM outside. Other non-transportable inhibitors showed asymmetry of different degrees (Baker et al., 1978).

Barnett et al. (1975), from their findings with 6-O-alkyl derivatives of galactose and glucose, have postulated that the reaction with the inside and the outside sites for transfer involves different ends of the glucose molecules. Thus glucose is seen to approach the outside facing binding site with its C-1 end, which on binding induces a conformational change in the carrier protein. The transformation of the protein into a second state allows association/dissociation from the inside side only. Hence the sites facing inside are only allowed to interact with the C-4, C-6 end of the glucose molecules.

Experiments with another inhibitor, Cytochalasin B, has provided further support for the above hypothesis. Cytochalasin B competitively inhibits 3-O-methyl glucose exchanges, while producing inhibition of the non-competitive type when measured by the Sen-Widdas (1962) method (Basketter and Widdas, 1978). This behaviour, they interpreted as due to Cytochalasin B reacting with

the internal site only. This observation and interpretation has been confirmed by Devés and Krupka (1978b). Further implications of the mode of action of Cytochalasin B are discussed in a later chapter.

That Cytochalasin B only binds on the internal sites of the hexose transfer system is consistent with the experiments by Lin and Spudich (1974) who showed that trypsin and pronase destroyed the Cytochalasin B binding sites when present on the inside, but has no effect outside. Masiak and LeFevre (1977) treated the internal and the external surfaces of erythrocytes separately with α-chymotrypsin and showed that with the proteolytic enzyme on the outside, the hexose transfer remained unaffected, but with it on the inside, there was a decrease in the hexose transfer activity. This suggests that the nature of the protein facing the outside is different from that facing inside. Widdas (1980) has referred to this as chemical asymmetry.

In conclusion, therefore, having discussed various aspects of the hexose transfer system, it must be stated that for a final answer as to the precise workings of this system, one may have to wait until the molecular structure and the actual mechanism of transfer are determined.

It is therefore the intention in this thesis to analyse and interpret all the kinetic data on the assumption that a simple asymmetric transfer system is responsible for the movement of sugars across the red cell membrane. The kinetics developed by Baker and Widdas (1973b) and, as

modified for analysing inhibition of exchange, by Baker et al. (1978) and by Basketter and Widdas (1978) were therefore used. Details are given in a later chapter.

Hexose Transfer System in Erythrocytes from Other Species

For a very long time, it seemed that the high permeability of glucose across erythrocyte membranes was a peculiarity of primates (Kozawa, 1914). The erythrocytes of no other animals blood readily available in the laboratory, such as rabbit, guinea-pig, ox, pig and sheep possess the hexose transfer system to a degree which is detectable by the osmotic methods used for human erythrocytes. The erythrocytes of these species possess the fast transfer rate only at the fetal stage and this is lost during neonatal life (Widdas, 1955). These findings have been confirmed and further investigated by various works.

In 1967, comparative studies by Augustin, Rohden and Hacker on newborn and adult rabbits showed that newborn rabbit erythrocytes transported 2-deoxy-D-glucose similar to those of the adult human. The hexose transfer system in the newborn rabbit erythrocyte was found to be made up of two components, one saturable and the other non-saturable. The ability of transporting glucose in these cells was seen to progressively decrease during post-natal development. It decreased by 3 orders of magnitude with a half-life of 6.5 days. The elimination of fetal erythrocytes from the circulation was thought to be the cause of this decrease in the ability to transfer sugar across its membrane.

Zeidler, Lee and Kim (1976) extended this comparative study by using erythrocytes from newborn and adult pigs, and also reticulocytes produced in the adult pig in response to phenylhydrazine. Their results showed that a carrier-mediated transfer system involved in the hexose transport was present in the newborn piglet erythrocyte. They also found the system to be present in reticulocytes from the adult pig. This was, however, lost when the reticulocytes matured to normal adult erythrocytes. The transport in the reticulocytes was found to be 2 to 3 orders of magnitude lower than that from the erythrocytes of a newborn. Zeidler et al. (1976) concluded that the transport system in the piglets was not unlike that found in the human erythrocytes, however they emphasised several distinct differences. These differences were in the transport rates and also in the absences of the trans-stimulation phenomena found in the adult human cells.

Another similar study was carried out by Lee et al. (1976) to compare the hexose transfer system from the newborn and adult dog erythrocytes. They investigated the transfer of sugar across young and old red blood cells from newborn dogs and also from phenylhydrazine induced reticulocytes in the adult dogs. They found the adult red cells to be much less permeable to 3-O-methyl glucose as compared to the cells from the newborn dog. Both the cells from the newborn and the adult dog showed saturation at high concentrations. They also found that the 3-O-methyl glucose uptake in the reticulocytes from the

adult animal was not much different from that of mature cells. Thus they concluded that the fetal cells in the newborn were different from induced reticulocytes in the adult dog.

Kim and Luthra (1977) showed that glucose transfer in naturally occurring reticulocytes from the newborn piglets was decreased within 3-4 days to a level of phenylhydrazine induced reticulocytes, in the adult pig. Moreover the reticulocytes in the adult animal lost their glucose transport ability in the course of maturation to red cells. They also showed that the change from permeable fetal cells to impermeable adult cells is brought about by two factors. These being the dilution and elimination of fetal cells in the circulation after birth. Their studies also revealed that the loss of facilitated glucose transport of red cells from the newborn pig was due to the animal producing progressively less glucose-permeable cells as gestation proceeded.

In a more recent comparison of the carrier-mediated transfer of sugars in humans and the fetal and newborn rabbit, it has been shown that the fetal and newborn rabbit red blood cells possess two different transfer systems. One being fast and Cytochalasin B sensitive and the other being slow and Cytochalasin B insensitive (Jung et al., 1980). The fast, Cytochalasin B sensitive system disappears as the rabbit ages. Thus only the slow, Cytochalasin B insensitive process is observed in the adult rabbit. The fast process is found to be similar

to that found in the human erythrocyte, not only in its speed but also in its sensitivity to chemical inhibitors such as Cytochalasin B and mercuric chloride.

Nucleated Avian erythrocytes are also permeable to glucose, however their permeability is much less than in the human erythrocytes (Mond, 1930; Höber and Ørskov, 1933, as cited by Kregenow (1977). Also the carrier-mediated transfer of glucose in the avian erythrocyte is accelerated by anoxia and is accompanied by a change in the sugar-carrier affinity (Wood and Morgan, 1969; Cheung et al., 1976). This carrier-mediated transfer system can also be accelerated by mercuric chloride.

The fetal guinea-pig red blood cells have also been compared with adult human red cells with respect to their hexose transfer system. The fetal and newborn guinea-pig possess a hexose transfer system which is similar in speed to that found in the human erythrocytes (Widdas, 1955). The pH dependency of these fetal-cells' glucose transfer system is similar to that of the human, whilst the temperature dependency shows a significant difference (Dawson and Widdas, 1964). The two half-saturation constants although similar at 37 ^{o}C, do not fall in the same manner as the temperature is decreased. On the other hand, the maximal transfer rate varies similarly for both the species.

Further comparisons of the action of various inhibitors on the hexose transfer system of red blood cells from feta- and newborn guinea-pig and of red blood cells from adult humans have been made during the course of work described in this thesis.

Post-natal Changes in Erythrocyte Membranes

The presence of a hexose transfer system in fetal erythrocytes in a number of mammals, similar to that found in adult human erythrocytes (Widdas, 1955) and its subsequent loss in adult life led workers to follow post-natal changes in these cells, with regards to the hexose transfer system.

The hexose transfer system in young guinea-pig red blood cells showed that the permeability constant (k') for glucose, for cells at two days post-partum was 0.058. This permeability constant dropped to 0.039 at nine days and to 0.027-0.012 on the 23rd day (Widdas, 1955). Widdas concluded that the fall in hexose permeability was due to a loss of the hexose permeable cells and also a dilution of the permeable cells with impermeable cells.

The work of Jung et al. (1980) who investigated the post-natal change in the hexose system in newborn rabbit erythrocytes has already been described. Since they found that the age-related loss of the fast glucose transfer site, which is Cytochalasin B sensitive and the glucose sensitive Cytochalasin B binding had similar decay constants and since these age-related losses were comparable with the mean life span of rabbit erythrocytes, they suggested that these two sites must occur in the same population of erythrocytes in the neonatal circulation. Jung et al. (1980) therefore concluded that the hexose transfer 'carrier' protein occurred transiently in fetal

rabbit erythrocytes and that this protein is responsible for the fast hexose transfer seen in these cells.

The age-related changes in erythrocyte activity are by no means isolated to the hexose transfer system and other age-related changes have been reviewed by Pennell (1964). Nucleoside permeability of fetal red cells in the sheep is much higher than in the adult sheep cells (Jarvis and Young, 1982). This high permeability to nucleosides is also a characteristic of human erythrocytes (see review by Plagemann and Wohlhueter, 1980). This is another facilitated transfer system which in these respects parallels the hexose transfer system described in this thesis.

Inhibitor Studies

Since, as for enzymes, the hexose transfer system possesses 'active-sites', these sites may be inhibited by various drugs, or chemicals. The study of the effects and mode of action of substrate analogues and related compounds, together with other chemicals has revealed a wide variety of information which in turn has provided a valuable insight into the mechanism of this transfer system.

It is vital to almost all experiments concerned with the mechanism of hexose transport across cell membranes, whether at the molecular or higher level, to have access to really specific inhibitors. It is also vital to be able to clearly define the mode of action of an inhibitor to be able to obtain any significant information about the system itself.

By analogy, we may classify the inhibitors of the hexose transfer system in classic enzymological terminology. Hence a brief outline of the classic behaviour of inhibitor shall be discussed here.

The activity of a system may be diminished by two main varieties of inhibitors, the competitive and the non-competitive inhibitors. The latter can either be reversible or irreversible processes. In irreversible inhibition, the inhibitor is covalently linked to the 'carrier-molecule', or it may be bound so tightly that its dissociation from its inhibitory site is very slow. Thus, an irreversible inhibitor would be characterised by a velocity constant for the inhibitory reaction and there would be a progressive increase of inhibition with time. Reversible inhibitors, on the other hand, are usually characterised by a rapid equilibrium of the inhibitor and the 'carrier', thus a definite degree of inhibition would be reached which is independent of time.

The simplest type of reversible inhibitor is exerted by a competitive inhibitor. A competitive inhibitor resembles the substrate and binds to the active site of the carrier. The substrate is thus prevented from binding to the same active site. That is to say, the binding of the substrate and a competitive inhibitor are mutually exclusive events. A competitive inhibitor therefore diminishes the rate of transport, in our case, by reducing the proportion of carriers that have a bound substrate.

In non-competitive inhibition, which can also be reversible, the inhibitor and the substrate can bind simultaneously to a carrier molecule. A non-competitive inhibitor acts by decreasing the turnover number of the carrier rather than diminishing the proportion of carrier molecules that have a bound substrate (see Dixon and Webb, 1964).

Furthermore, competitive inhibitors can be distinguished from non-competitive inhibitors by the fact that competitive inhibition is abolished by high substrate concentration, however, the effect of a non-competitive inhibitor is maintained at all substrate concentrations.

Thus the affinity and the quantity parameters analogous to the classical enzymatic K_m and V_{max} for various experimental procedures and also the K_i for various inhibitors can be used confidently to compare similar systems from different species.

Present Problem

In spite of the extensive studies on the hexose transfer system in the human, which have all tended to confirm the presence of a specific component, 'a carrier' within the membrane, comparatively little is known about this function in lower mammals. Some studies have been carried out to detect the presence of the carrier function in various animals, however few studies have so far been made to compare the sensitivity of the various systems to inhibitors of the hexose transfer system.

The present project was undertaken to make a comparative study of the hexose transfer system in fetal and newborn guinea-pig red blood cells, since, the facilitated transfer system in these cells is just as fast, if not faster, than that found in human erythrocytes.

Although it is fully appreciated that the morphological differences between the bloods of an adult human and of a developing guinea-pig fetus must be quite significant, it is also appreciated that it may be assumed that the functional components in both these bloods must serve adequately at their appropriate stage in development. Yet the resemblance in the hexose transfer system in the human and the fetal and newborn guinea-pig is so great that a comparision seems justified.

An attempt has therefore been made to compare the hexose transfer systems of the human and fetal and newborn guinea-pig by making use of chemicals which are either analogues of glucose or in some way react with the membrane component responsible for the transport process.

Firstly, the non-transportable inhibitors, 4,6-O-ethylidene-α-D-glucopyranose (ethylidene glucose) and methyl-2,3-di-O-methyl-α-D-glucopyranoside (trimethyl glucoside) have been used to look at the degree of asymmetry between the inside and the outside sites of the transport system.

Secondly, Cytochalasin B has been used in two ways. Primarily this has been used to compare the asymmetry of affinity between the two faces of the membrane, remembering

the fact that Cytochalasin B is assumed to be a competitive inhibitor for the internal site only. The Cytochalasin B has also been used to estimate the possible number of sugar binding sites on the red blood cell from the newborn guinea-pig.

Thirdly, a biphenolic group of compounds, which potently inhibit the hexose transfer system have been used, again in the hope that they may provide valuable information on the asymmetry of the transfer system. This group of compounds is comprised of phloretin, phlorizin and polyphloretin phosphate.

Finally, the non-competitive, irreversible inhibitors, 2,4-Dinitrofluoro-benzene and 2,4-Dinitrobromobenzene have been utilised, to obtain some clues as to how the hexose transfer system of fetal and newborn guinea-pig red cells compares with that of the adult human erythrocytes.

CHAPTER 2

METHODS AND MATERIALS

Materials

1. Blood
2. Sugars
3. Inhibitors
4. Scintillator materials
5. Chromatographic materials
6. Anaesthetic
7. Miscellaneous

Methods

1. Procedure for preparation of solutions
2. Procedure for blood collecting and washing
3. Procedure for glucose exits
4. Procedure for equilibrium exchanges
5. Procedure for estimating the number of hexose transfer sites in the erythroctye membrane
6. Procedure for FDNB and BrDNB inhibition
7. Procedure for osmotic balancing
8. Procedure for chromatographic purification of ethylidene glucose

Materials

1. Blood

The human, fetal guinea-pig and newborn guinea-pig blood was obtained as described later in this chapter. The anticoagulant heparin, was from British Drug Houses Ltd.

2. Sugars

D-glucose was supplied by May & Baker Ltd.
2-deoxy-D-glucose and 3-O-methyl glucose were both supplied by Koch-Light Laboratories Ltd.

The radioactive 3-O-methyl glucose was obtained as 3-O-methyl [U-^{14}C] glucose, specific activity 379 μCi/mg, in aqueous solution containing 3% ethanol, contained in a "Duoseal" vial from the Radiochemical Centre, Amersham.

3. Inhibitors

1. 1-Fluoro-2,4-dinitrobenzene (FDNB) was from British Drug Houses Ltd.
2. 1-Bromo-2,4-dinitrobenzene (BrDNB) was from British Drug Houses Ltd.
3. Phloretin was from ICN Pharmaceuticals, Inc. Life Sciences Group, Plainview, N.Y.
4. Phlorizin was from Sigma Chemical Company.
5. Polyphloretin phosphate as a sodium salt, average MW 4600, was from Leo, Hälsingborg.
6. Cytochalasin B was from Aldrich Chemical Co., Inc.

The radioactive Cytochalasin B was obtained in the

form [4(n)-^{3}H] Cytochalasin B, specific activity 21.5 mCi/mg, in ethanol in borosilicate multidose vials with additional screw cap ("Duoseal" vials), sealed under nitrogen from the Radiochemical Centre, Amersham.

7. 4,6-O-Ethylidene-α-D-glucopyranose (Ethylidene glucose) was from Koch-Light Laboratories Ltd.
8. Methyl-2,3-di-O-methyl-α-D-glucopyranoside (Trimethyl glucoside) was from Koch-Light Laboratories Ltd.
9. Mercuric chloride was from May & Baker Ltd.

4. Scintillator Materials

1,3-Diphenyloxazole (PPO) was from Packard.
1,4-Di[2-(5-phenyloxazolyl]benzene (POPOP) was from Koch-Light Laboratories Ltd.
Toluene was from British Drug Houses Ltd.
Triton-X100 was from Koch-Light Laboratories Ltd.
Multisol II was from Intertechnique, France.
Scintillator 299^{TM} was from Packard.

5. Chromatographic Materials

Whatman 3M chromatography paper was from W & R Balston Ltd.
Propan-2-ol was from British Drug Houses Ltd.
Acetone was from British Drug Houses Ltd.
Aniline was from British Drug Houses Ltd.
Ortho-phthalic acid was from British Drug Houses Ltd.
Butan-1-ol was from British Drug Houses Ltd.

6. Anaesthetic

Nembutal was from Abbott Laboratories Ltd.

7. Miscellaneous

Sodium chloride was from British Drug Houses Ltd.

Potassium iodide was from May & Baker Ltd.

Sodium hydroxide was from British Drug Houses Ltd.

Sodium dihydrogen orthophosphate was from British Drug Houses Ltd.

Absolute alcohol was from British Drug Houses Ltd.

Inositol (meso) was from British Drug Houses Ltd.

Malonamide was from British Drug Houses Ltd.

Hyamine 10-X hydroxide 10% w/v in methanol was from British Drug Houses Ltd.

Potassium chloride was from British Drug Houses Ltd.

Sodium Tauroglycocholate (from ox bile) was from British Drug Houses Ltd.

1. Procedure for Preparation of Solutions

A. Phosphate buffered saline

Phosphate buffered saline has been used throughout this study. It has been used to wash the cells, make up sugar solution and also all the inhibitor solutions. The phosphate buffered saline was prepared fresh prior to each experiment from stock solutions of phosphate buffer, sodium hydroxide, sodium chloride and potassium chloride.

The 0.2 M stock phosphate buffer solution was prepared by dissolving 31.2 g sodium dihydrogen orthophosphate in almost 1 litre of deionised distilled water, followed by the addition of 2 M sodium hydroxide, sufficient to bring the pH to 7.4. The stock solution was finally made up to 1 litre with further addition of deionised distilled water.

Prior to each experiment, the phosphate buffered saline was prepared by diluting 50 ml phosphate buffer stock solution with 35 ml of 10% sodium chloride and 2.5 ml 1 M potassium chloride to 500 ml with deionised distilled water, and if necessary, the pH of the buffered saline was adjusted to 7.4 by dropwise addition of 2M sodium hydroxide.

B. Stopping solution

Stopping solution is the name given to a solution designed to inactivate the hexose transfer system in the

erythrocyte membrane, instantaneously. This is achieved by the presence of powerful inhibitors, phloretin and mercuric chloride, in a saline medium, which during experimentation was cooled to ice temperature.

The stopping solution was made up of 27.2 mg phloretin dissolved in 10 ml absolute alcohol plus 540 mg mercuric chloride, 112.5 mg potassium iodide and 20 g sodium chloride in 1 litre deionised distilled water.

C. Scintillant

The scintillant used for radioactive counting on a spectrometer was made up so that one could use up to 5% water in ethanol, without separation of components.

The toluene based scintillant was made up by dissolving 4.0 g 1,3-Diphenyloxazole and 0.2 g 1,4-Di[2-(5-phenyloxazolyl]benzene in 667 ml toluene and 333 ml Triton-X100.

For aqueous samples, an industrially produced scintillant, Multisol II, was used. Also another industrial scintillator, scintillator 299^{TM}, based on xylene was used.

D. Sugar and Inhibitor solutions

All sugar solutions were prepared in phosphate buffered saline.

FDNB, BrDNB, phloretin and phlorizin solutions were prepared by dissolving these in a minimum of absolute

alcohol before each compound was made up to the desired concentration with phosphate buffered saline.

A stock solution of 0.1 mM Cytochalasin B was prepared by dissolving 2.4 mg of the compound into 50 ml absolute alcohol and subsequent concentrations were made by diluting appropriate volumes of this alcoholic solution with appropriate volumes of phosphate buffered saline.

For all optical experiments, the solutions were first filtered through Whatman No 50 papers to remove all traces of dust particles, which would otherwise interfere with the quality of recording.

2. Procedure for Blood Collecting and Washing

Human

The human blood was obtained by venepuncture and collected into centrifuge tubes containing the anticoagulant heparin. The required quantity of blood was suspended in 5 ml buffered saline at pH 7.4 and centrifuged at 3000rpm for 4 minutes. The buffy coat was then removed and the red cells washed twice more with the buffered saline, after which the cells were ready for use in experiments.

Normally fresh human blood was used for each experiment, and no blood more than one day old was used.

Guinea-Pig

The guinea-pig blood was obtained either from near full-term fetuses or recently newborn guinea-pigs. The gestation age of the fetal guinea pigs was estimated using the formula:

$$W^{1/3} = a(t-t_o)$$

which for guinea-pigs is

$$W^{1/3} = 0.09\ (t-16)$$

where W is the weight in grams, a is the specific fetal growth velocity, t is the time since conception and t_o is an estimated time, depending on the gestation period. (For a further explanation of the formula, see Huggett and Widdas, 1951).

Fetal guinea pigs

The fetal blood was obtained from near full-term fetuses following the procedure described by Dawson and Widdas (1964).

Under nembutal anaesthesia and ceasarean-section, the fetuses were exposed and blood obtained from the umbilical vein with a syringe and needle, heparin being used to prevent coagulation. The fetal blood was then washed in a similar manner to human blood, before experimentation.

Recently newborn guinea-pigs

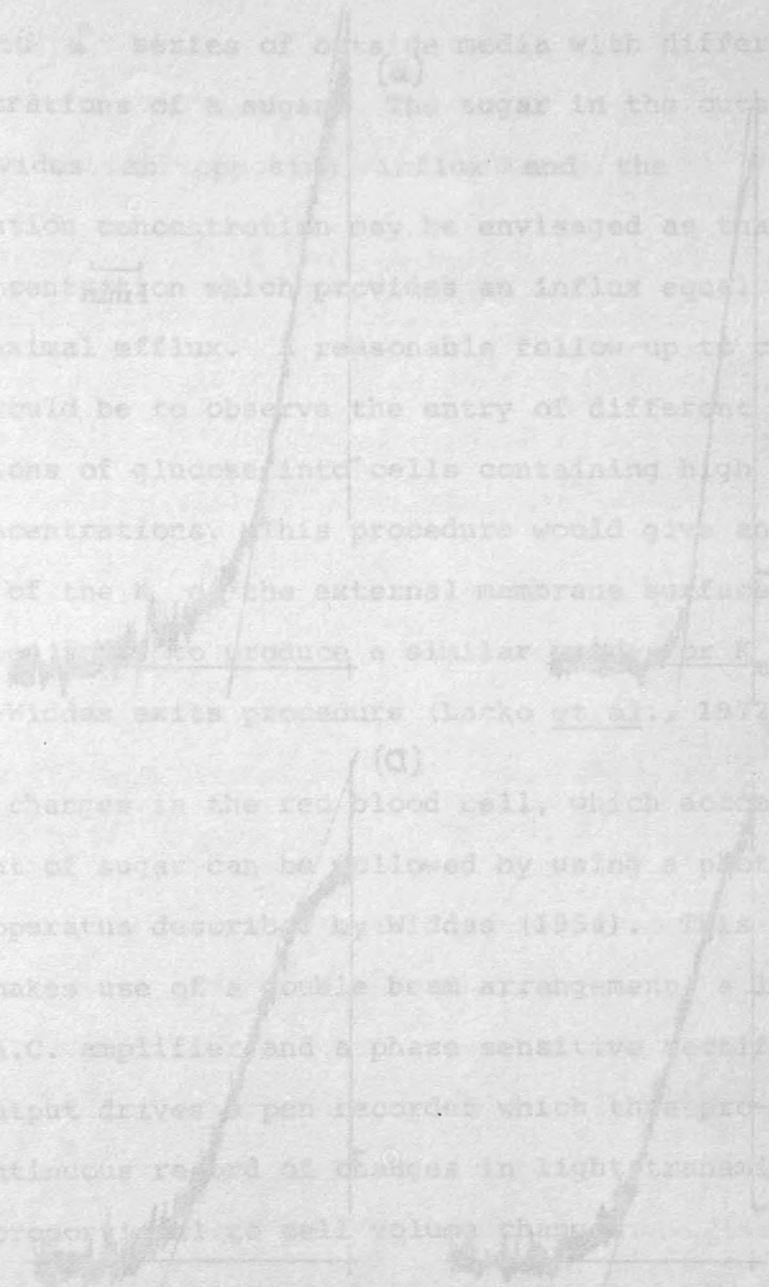
The newborn guinea-pigs were stunned with a quick blow on the head and bled by slitting the throat. The blood was thus collected, from the vessels in the neck, into centrifuge tubes containing a small quantity of buffered saline

with heparin. This procedure allowed a rapid mixing of the heparin with the blood to prevent blood clots forming, which often occurred if no buffer was present. The blood was then filtered through glass wool to remove any fur that may be present from the bleeding procedure. Once again, the blood was washed as for human cells before experimentation.

3. Procedure for Glucose Exits

These are based on the unique properties of the erythrocytes to act as osmometers in a limited volume range and also the fact that the presence of a high concentration of hemoglobin within the cell allows photoelectric measurements, reflecting volume, to be made relatively rapidly and accurately.

Sen and Widdas (1962a, b) described a procedure for exits where the concentration of a substrate is set at a limitingly high level on the inside of the red blood cell and the movement of the substrate is into a medium containing various concentrations of the substrate and/or inhibitor . Thus on resuspension of red blood cells containing a high sugar concentration into buffered saline solutions containing low sugar concentrations, the cells first osmotically swell and then shrink as the exodus of the sugar proceeds. With this high concentration of sugar inside, the internal sites of the hexose transfer system are saturated, hence for around two thirds of the total sugar exit, the rate of exit is constant and is maximal at zero outside concentration.


(A)
(B)
(C)
(D)

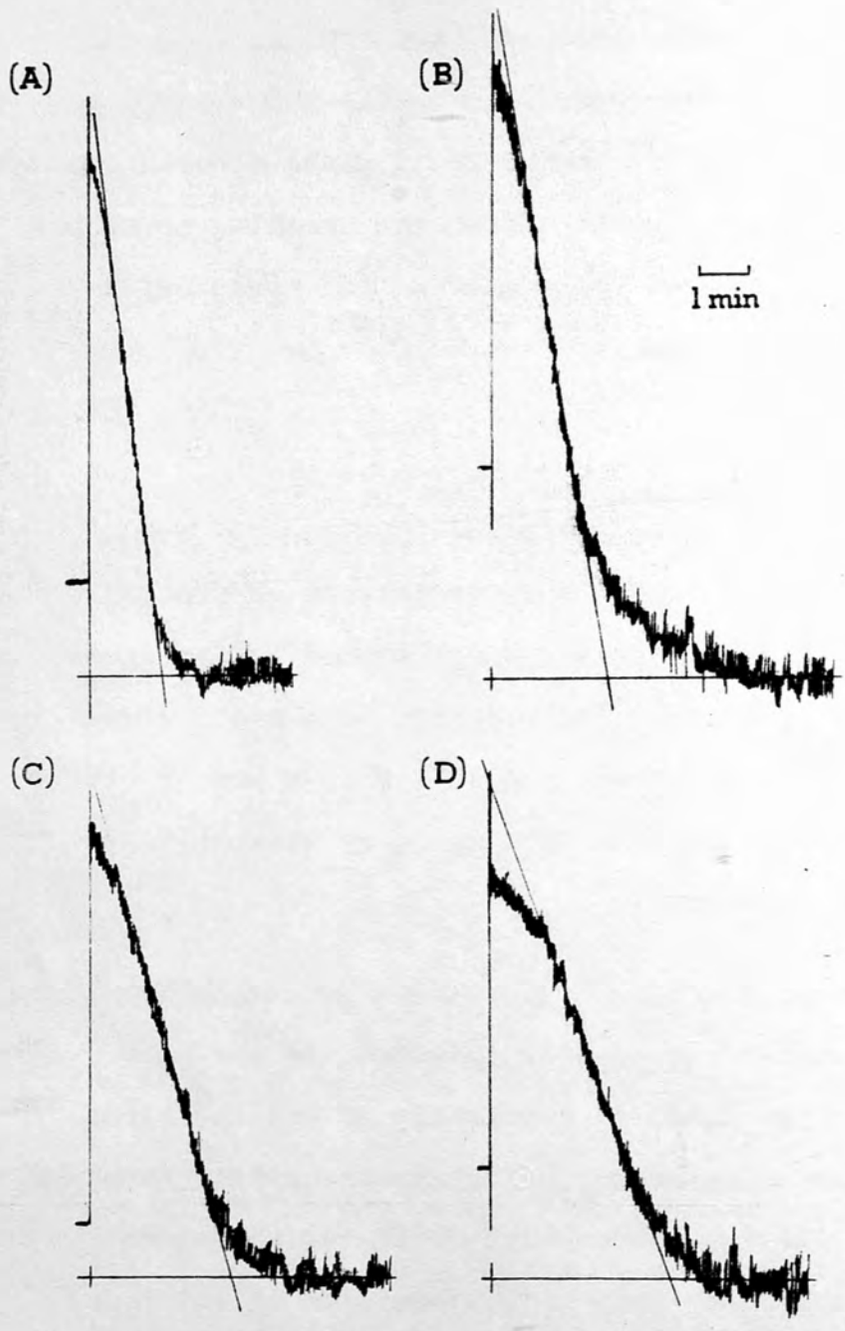


Figure 2.1

Typical traces of the exit of glucose from fetal and newborn guinea-pig erythrocytes preloaded to contain 76 mM glucose, into 1.1 mM glucose (A), 3.0 mM glucose (B), 4.9 mM (C) and 6.8 mM glucose (D). The linear part of each trace is produced to cut the base line and the time from the moment of injection of cells to this intersection represents the 'exit time'.

This method, therefore, involves following a saturated efflux into a series of outside media with different low concentrations of a sugar. The sugar in the outside medium provides an opposing influx and the half-saturation concentration may be envisaged as that outside concentration which provides an influx equal to half the maximal efflux. A reasonable follow-up to confirm this would be to observe the entry of different concentrations of glucose into cells containing high glucose concentrations. This procedure would give an estimation of the K_m of the external membrane surface. This has been found to produce a similar value for K_m as the Sen-Widdas exits procedure (Lacko et al., 1972).

The volume changes in the red blood cell, which accompany the movement of sugar can be followed by using a photoelectric apparatus described by Widdas (1954). This apparatus makes use of a double beam arrangement, a low frequency A.C. amplifier and a phase sensitive rectifier. The D.C. output drives a pen recorder which thus produces a continuous record of changes in light transmission which are proportional to cell volume changes.

A typical trace of 76 mM glucose exits into low sugar concentrations is shown in Figure 2.1.

Procedure

To load the red blood cells with 76 mM glucose, the washed packed cells were suspended in phosphate buffered saline containing glucose and incubated at 37 ^{o}C for 30 minutes. The incubation medium contained

10.1 ml phosphate buffered saline, 0.5 ml 30% glucose, 0.2 ml 2 x 10^{-3} w/v bile salts and 0.2 ml packed cells. Prior to experiment, this cell suspension was cooled to appropriate temperatures for exit measurements.

For the measurement of exits, ca. 0.15 ml cell suspension (containing less than 3 µl cells) was rapidly injected into a cuvette containing 21 ml buffered saline with either different low concentrations of glucose or different concentrations of both sugar and inhibitor.

The contents of the cuvette and all the stock solutions used during the experiment were maintained at constant temperature. The cell suspension in the cuvette was continually stirred throughout each measurement to prevent sedimentation of the cells.

All solutions were filtered before any measurements were made to avoid light scatter due to dust particles etc. The first injection of the cell suspension was normally made into 21 ml buffered saline containing 1 ml of the 30% glucose solution diluted by a factor of 20, since this allowed sufficient time for the initial adjustment of the shutter on the apparatus which was necessary to balance the two light beams.

Theoretical treatment of the hexose transfer kinetics predicts that the time as measured from the moment of injection to the point where the base line, representing the final equilibrium volume, was cut by the line produced by extrapolating the linear part of the exit record (see Figure 2.1), should be a linear function of

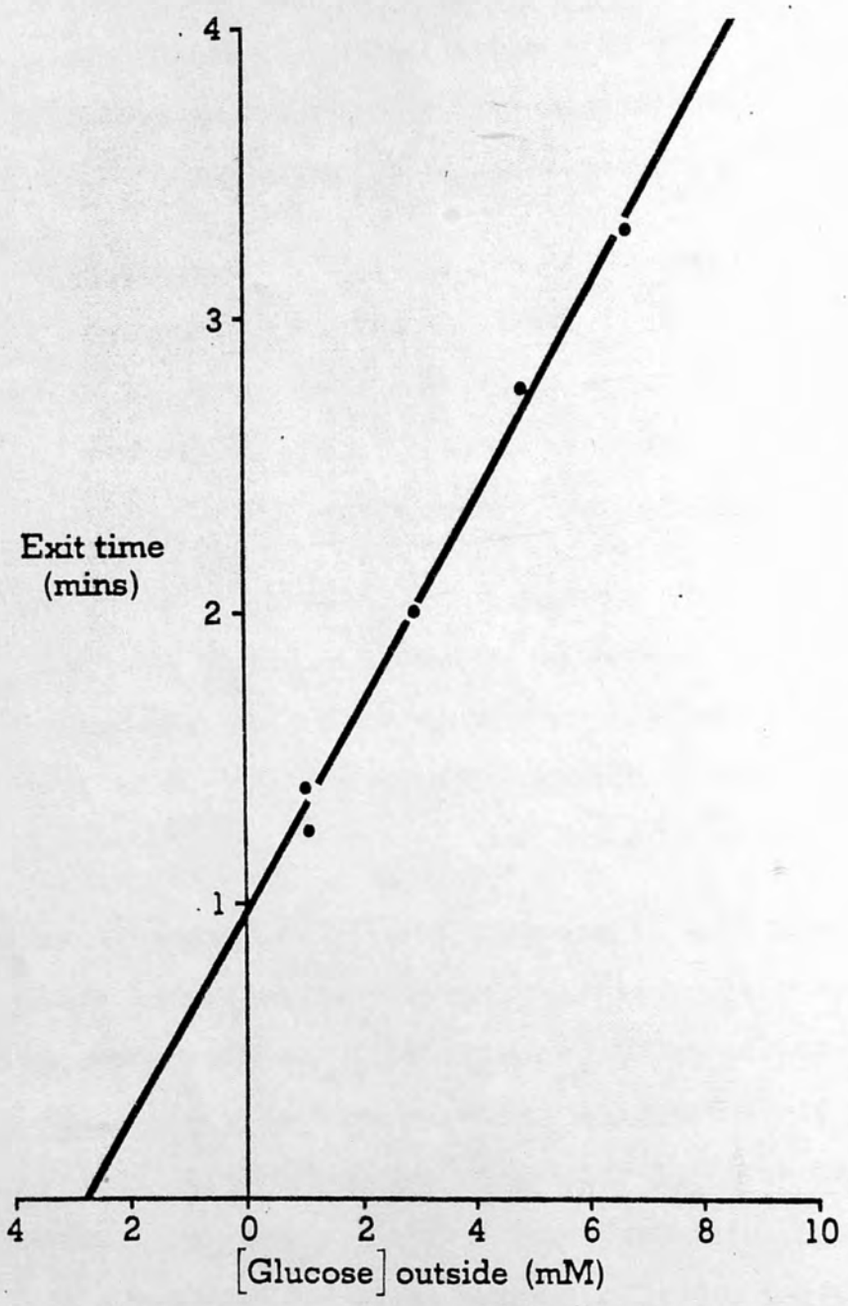


Figure 2.2

Exit time of 76 mM glucose into increasing concentrations of glucose in the outside medium, at 16 °C. The exit times were obtained from records such as shown in Figure 2.1. The intercept on the ordinate represents time which would have been taken for exits into a glucose-free medium and the intercept on the abscissa gives the concentration of glucose into which the exit time would be twice that into a glucose-free medium.

the outside glucose concentration. Thus a plot of exit times versus the outside glucose concentration should give a straight line graph. The value of the intercept on the abscissa corresponds to the half-saturation concentration and the intercept on the ordinate gives the time for exit into sugar free medium which is inversely proportional to the maximal velocity of glucose efflux. A typical plot of exit times versus the glucose concentration, for fetal and newborn guinea-pigs is shown in Figure 2.2.

A recent modification in the kinetic interpretation of the hexose transfer system takes into account asymmetry (Widdas, 1980). The Sen-Widdas exit technique can be visualised as giving the half-saturation concentration for the outside site only. The half-saturation concentration is typically increased by competitive inhibitors but will only show an increase in this constant if the inhibitor interacts with the outside site; it will not show this increase if the action of the inhibitor is restricted to the interior of the cell.

For the determination of K_I for an inhibitor, the exit time of glucose into glucose free medium containing various inhibitor concentrations is plotted against the inhibitor concentration. The intercept on the abscissa is equal to the apparent K_I for the inhibitor. Although using a glucose free medium, the 0.15 ml suspension of cells added to the cuvette produces a finite outside glucose concentration, but this can be allowed for in calculating the true K_I (Forsling and Widdas, 1968).

The advantages of using this procedure to estimate the half-saturation concentrations for the hexose transfer system are many fold. The most apparent is that this is a quick and a relatively easy procedure. It can be readily adapted to different temperatures, thus measurements of kinetic parameters at low temperatures are possible. Another important consideration in the choice of this procedure is that of simplicity. That is to say that since only one sugar is used, the complication of competition is eliminated, also since the cells are suspended in a large volume, there is a negligible change in concentration produced by the efflux of glucose into the outside medium.

4. Procedure for Equilibrium Exchanges

A procedure which allows measurements of fluxes into and out of cells, with both the internal and external medium arranged to be at electrochemical equilibrium, is obviously desirable. The use of radioactive isotope tracer sugar molecules makes such a procedure a practical proposition. Since under these conditions there is no net flow of sugar, one measures only unidirectional fluxes.

The loss or gain of radioactivity under the above conditions may be expected to rise or fall exponentially to the equilibrium value. This may be expressed as follows:

$$-2.303 \log(C_t - C_\infty) = kt + \text{constant}$$

where C_t is the radioactivity associated with an aliquot of cells at time t. C_∞ is the radioactivity for a sample

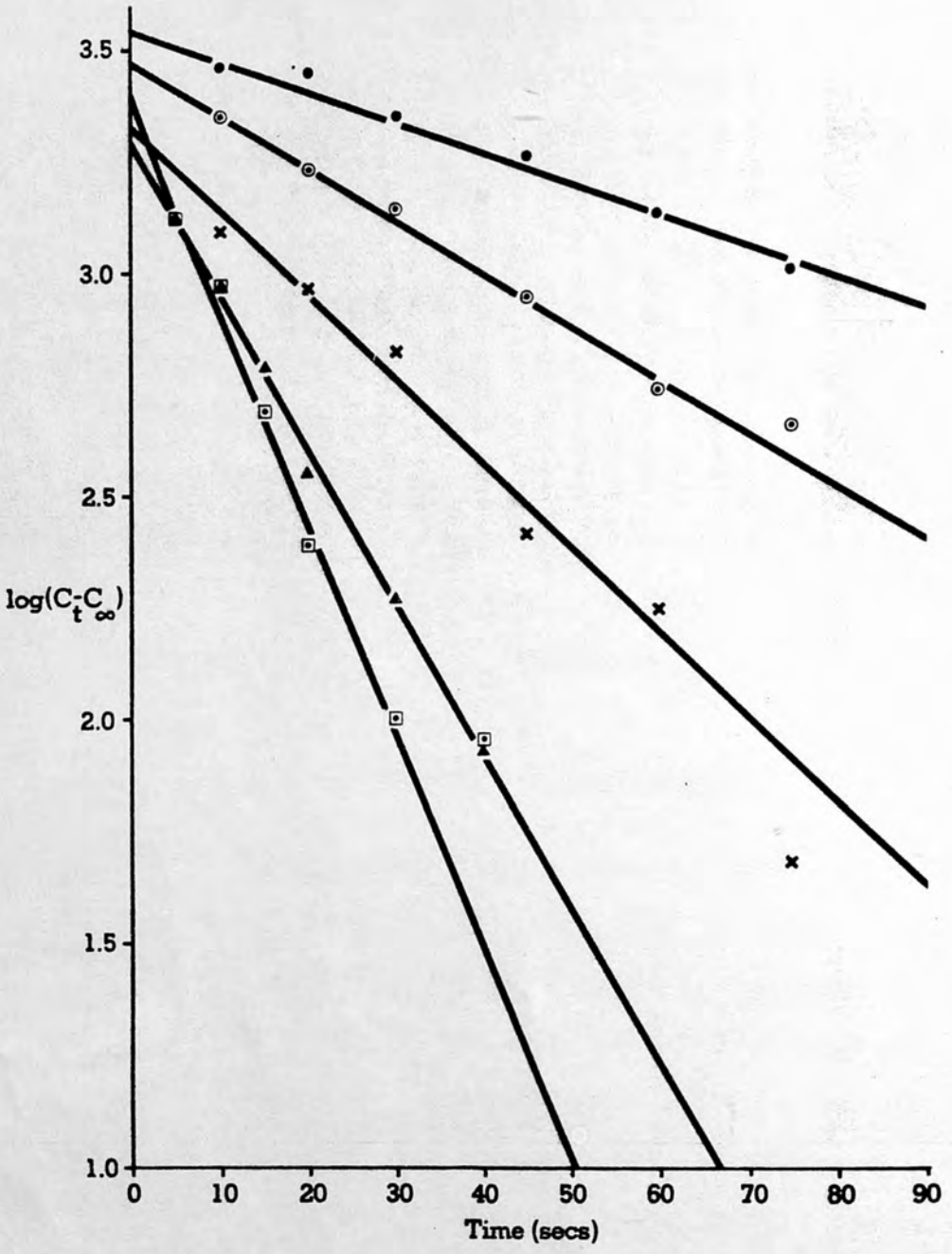


Figure 2.3

The loss of intracellular radioactivity during 3-O-methyl glucose exchanges with fetal and newborn guinea-pig erythrocytes, at 16°C at different concentrations plotted logarithmically. Points ●, 160 mM exchanges; points ⊙, 80 mM exchanges; points ×, 40 mM exchanges; points ▲, 10 mM exchanges and points ⊡, 2 mM exchanges. The slopes of such lines were used to derive the exchange flux as described in the text.

at equilibrium. Thus the slope of a plot of the logarithmic term versus the time (multiplied by 2.303) would give the constant k and the flux J is given by J = kC where C is the concentration of sugar, see Figure 2.3.

Because of the small volume of cells compared to the suspending medium, the loss or gain in the specific activity of the medium is negligible. Also by subtracting the equilibrium counts from counts at time t, the error that may be due to the extracellular fluid trapped in the cell pellets is eliminated.

Thus this technique for measuring equilibrium exchange fluxes can be utilized to measure sugar fluxes at different concentrations and different inhibitor concentrations to enable one to estimate the affinity constant for sugars and affinity constants for various inhibitors of the hexose transfer system. (For further details see Miller, 1968a; Eilam and Stein, 1972; Lacko et al., 1972 and Eilam, 1975).

Procedure

Packed, washed cells were incubated in buffered saline with appropriate amounts of 3-O-methyl glucose and trace amounts of ^{14}C-3-O-methyl glucose at 37 ^{o}C for 30 minutes. The cell suspension was cooled to 16 ^{o}C in most cases before fluxes were measured.

Following incubation, the cells were separated from the medium by centrifugation and these packed cells were resuspended, at time zero, into 20 ml corresponding

exchange medium, contained in a conical flask surrounded by a water jacket to maintain temperature control. The contents of the flask were continually stirred by a magnetic stirrer. At set intervals, 1.0 ml samples of the cell suspension were withdrawn, using an automatic syringe and injected into a conical centrifuge tube containing 10 ml ice-cold stopping solution. Thus successive samples were taken at known times and two at infinite time, when the radioactive tracer had equilibrated. The conical tubes with 'stopped' cells were centrifuged at 4000 rpm for 4 minutes, the supernatant discarded and the cell pellet washed with a further 2 ml stopping solution and recentrifuged. Finally the supernatant was completely discarded by suction. To the cell pellet was then added 50 µl deionised distilled water and the pellet broken down by mechanical stirring followed by 1.2 ml absolute alcohol. After a final centrifugation of this alcoholic suspension, 1.0 ml of the supernatant was placed in 10 ml scintillation liquid for radioactive counting. All counts were made using the Packard scintillation spectrometer.

The advantages of this procedure are that the exchange flux, whether measuring efflux or influx, is the same, thus for all experiments reported here, the efflux was measured as this was the more convenient. Also the fact that the fluxes were measured without the presence of any osmotic effects was of course an advantage.

5. Procedure for Estimation of Numbers of Hexose Transfer Sites in the Erythrocyte Membrane

Based on the assumption that there is a one to one relationship between Cytochalasin B binding and the hexose transfer sites which are inhibited, an estimation of the number of hexose transfer sites in the erythrocyte membrane is possible.

The quantity of cells in a sample, in which binding is to be estimated, can be determined by absorbance measurements and comparing these with absorbances of standards. The quantity of bound Cytochalasin B in these cells can be measured radioactively, using ^{3}H-Cytochalasin B.

There is a saturable and an unsaturable uptake of Cytochalasin B by erythrocytes and the total uptake would take the following form:

$$\text{total uptake} = X\left[\frac{I}{I + \emptyset_I}\right] + YI$$

where X is the saturable and Y the linear constants representing Cytochalasin B uptake, I is the Cytochalasin B concentration and $\emptyset_I$ is the apparent half saturation concentration of Cytochalasin B.

To determine $\emptyset_I$, the inhibition of exchange has to be measured at each of the sugar concentrations used. In the absence of sugar, the $\emptyset_I$ used was that estimated from a Lineweaver-Burk plot of 1/flux against 1/sugar concentration in the presence of a fixed concentration of inhibitor. (See figure 5.5).

The mean saturable uptake correlated with the inhibition of exchange, corresponds to the concentration of hexose transfer sites in the erythrocyte membranes (Basketter and Widdas, 1978).

Procedure

The inhibition of 2 mM and 20 mM 3-O-methyl glucose exchange fluxes was measured at 16 ^{o}C, at 0.05, 0.25, 0.5 and 1.0 μM Cytochalasin B, the concentrations at which the uptake of this inhibitor was also to be measured.

The number of red blood cells was estimated indirectly by measuring the absorbance at 393 nm. The hemoglobin standards were prepared by first diluting 0.1 ml whole blood and washed packed cells to 10 ml with deionised distilled water respectively. 0.5 ml of each of these solutions was further diluted with 9.4 ml deionised distilled water and 0.1 ml hyamine hydroxide added and the absorbance measured.

Cytochalasin B uptake was estimated at different inhibitor concentrations. The cells were incubated in a similar manner described earlier for measurements of inhibition of equilibrium exchange fluxes. During the measurement of equilibrium exchange fluxes, 1.0 ml samples of the cell suspension were taken at 100 seconds and 110 seconds, from the start of the experimental run. These samples were centrifuged on an ultrafuge for 1 minute. The supernatant was collected and stored for subsequent radioactive counting. To the cell pellets

from each of these samples was added 0.2 ml hyamine hydroxide to dissolve the pellets. 0.1 ml of these solubilized pellets was treated with 0.2 ml hydrogen peroxide until it became colourless, then a few drops of sulphuric acid were added to destroy the excess peroxide which would otherwise interfere with scintillation counting. The colourless, solubilized pellets were then transferred to vials with Multisol II, a scintillating liquid for counting aqueous samples. A further 0.05 ml of the hyamine hydroxide solubilized pellet was added to 9.95 ml distilled water and this was used for absorbance measurements to estimate the cell content in these pellets.

The 3-O-methyl glucose fluxes were measured using a ^{14}C-labelled form of the sugar and the Cytochalasin B was estimated by the ^{3}H-labelled form of the inhibitor. $^{14}Carbon$ and Tritium channels were selected on the scintillation spectrometer to minimise the interference of one count by the other.

6. Procedure for FDNB and BrDNB Inhibition

To inhibit the cells with these irreversible inhibitors, the procedure described by Bowyer and Widdas (1958) was followed. This procedure involved incubating the red blood cells with the inhibitor for a set time at a controlled temperature. The stock inhibitor solution was made up to be 2.37 mM, by first dissolving 44 mg FDNB or 58.2 mg BrDNB respectively in 11.4 ml absolute alcohol and then making up to 100 ml with buffered saline.

For experiments where the inhibition was carried out in the presence of a sugar, 2.0 ml washed packed cells were suspended in 21 ml buffered saline containing 698 mg 2-Deoxyglucose and incubated at 37 ^{o}C for 15 minutes to allow sugar equilibration. This cell suspension was then cooled to 22 ^{o}C and 33 ml DNFB (or BrDNB) also at 22 ^{o}C was added and the cell suspension reincubated, this time at 22 ^{o}C for 15 minutes. At the end of this period, the cell suspension was centrifuged at 3000 rpm for 4 minutes. The cells were resuspended in buffered saline and reincubated for 5 minutes to wash away the 2-Deoxy-glucose and the excess inhibitor. The washing procedure was repeated once again before the cells were used to either measure equilibrium exchange fluxes or glucose exits.

For experiments where inhibition was to be carried out in the absence of any sugar, 2.0 ml washed packed cells were suspended in sugar-free buffered saline and treated in the above manner from then on.

7. Procedure for Osmotic Balancing

Experiments involving nontransportable inhibitors, with upto 200 mM ethylidene glucose on the inside of the cells, called for a balancing of the osmotic effect it produced. The method described by Baker and Widdas (1973b) has been used here. This involves the use of inositol and malon-amide. With 200 mM ethylidene glucose on the inside, one third of the total osmotic balance in the outside medium

Table IIi

Quantities of malonamide and inositol used to balance osmotic difference brought about by various concentrations of non-transportable inhibitors, either when outside or when inside the cells.

Ethylidene glucose	Inside		Outside	
	Malon-amide	Inositol	Malon-amide	Inositol
10 mM outside	10 mM	-	-	-
50 mM outside	50 mM	-	-	-
50 mM inside	-	-	16.7 mM	33.3 mM
200 mM inside	-	-	66.7 mM	133.3 mM

was provided by malonamide and the other two thirds was provided by inositol. In the cases where high concentrations of ethylidene glucose on the outside were used, the cells were preincubated with malonamide.

Neither malonamide nor inositol affects the hexose transfer system in any way, but malonamide penetrates the red blood cell by simple diffusion at about three times the rate of ethylidene glucose.

Osmotic balancing was achieved by choosing the concentration of malonamide, in the outside medium, for which the penetration into the cells, during the sampling period, would correspond to the loss of ethylidene glucose from inside the cells. Consequently the cells remained in osmotic balance over an experimental run.

The quantities of malonamide and inositol used for different concentrations of ethylidene glucose are summarised in Table IIi.

8. Procedure for Chromatographic Purification of Ethylidene Glucose

The commercial grade ethylidene glucose was found to be contaminated with glucose and it was, therefore, necessary to first purify this before experimentation. The purification was carried out by preparative paper chromatography, using Whatman 3M paper. This was based on a suggestion by Barnett to Baker et al. (1978).

The ethylidene glucose, 1 g dissolved in 2 ml distilled water, was applied in a 25 cm long streak across the chromatographic paper, the streak being not more than 1 cm wide. The chromatogram was then run for around 16 hours with a 4:1 v/v propan-2-ol and water mixture. After this, an edge strip of the chromatogram was stained with aniline phthalate and heated at 70 ^{o}C for 15 minutes. The R_f value for glucose is about 0.5 whereas the ethylidene glucose runs near the solvent front.

The strip of paper containing only the ethylidene glucose was cut and eluted with water. The purified ethylidene glucose was then obtained by vacuum drying at 40 ^{o}C.

The aniline phthalate reagent was prepared by dissolving 0.93 g of aniline and 1.66 g of ortho-phthalic acid in 100 ml of water saturated butan-1-ol. The sprayed chromatogram strip was developed by heating at 105 ^{o}C for 10 minutes.

CHAPTER 3

KINETIC PARAMETERS OF THE HEXOSE TRANSFER SYSTEM IN THE FETAL AND NEWBORN GUINEA-PIG RED BLOOD CELLS

Results

Kinetic Parameters of the Hexose Transfer System in the Fetal and Newborn Guinea-pig red blood cells

A prerequisite of any kinetic study would be to characterise, as far as possible, the system under investigation.

The hexose transfer system in the erythrocytes in its simple form can be primarily characterised by two parameters. One parameter defines the affinity between the carrier and its substrate, whilst the other parameter describes the system's capacity for transfer activity.

The early quantitative data on the permeability of red blood cells to various solutes was obtained from techniques involving measurements of time taken for red blood cells to haemolyse, once they were suspended in media whose osmotic constituent was the compound under investigation. The penetration of this compound produced osmotic swelling of the red blood cells, leading to haemolysis. (Gryns, 1896; Kozawa, 1914; Jacobs et al., 1935; Jacobs et al., 1937).

Ørskŏv (1935) introduced a technique which did not involve the destruction of the red blood cells under test, but measured the volume changes of these cells which accompanied the penetration or net exodus of solutes.

The emergence of radioactively labelled sugars permitted the development of techniques, whereby sugar fluxes under equilibrium conditions could be measured. Thus, one is now able to measure equilibrium exchange fluxes as well

as fluxes with other combinations of inside and outside media.

In the present study, two techniques for measuring the affinity and the activity parameters of the hexose transfer system have been used. The Sen-Widdas exits technique (Sen and Widdas, 1962a, b), based on the Ørskŏv type of optical apparatus and the equilibrium exchange procedure, which utilizes radioactively labelled sugars, have been used. The advantages and disadvantages of these procedures have been discussed earlier.

The interpretation of all kinetic data has been based on the simple asymmetric kinetics described by Widdas (1980). A brief outline of the relevant components shall be given here, however the text should be referred to for a fuller explanation.

The transfer of sugars from side 1 to side 2 is considered to occur when the transfer site on side 1 is saturated and that on side 2 is unsaturated. The probability of saturation or unsaturation of a side is presumed to be the same as the saturation or unsaturation fraction of all components on that side. Thus, these probabilities are represented by $C_1/(C_1 + \emptyset)$ for side 1 and $a\emptyset/(C_2 + a\emptyset)$ for side 2, where $\emptyset$ and $a\emptyset$ are the half-saturation concentrations, a is the asymmetry factor and C_1 and C_2 are side 1 and side 2 sugar concentrations respectively.

The probability of the occurrence of transfer is given by

$$\underset{1 \rightarrow 2}{\text{Transfer}} = V_1 \frac{C_1}{C_1 + \emptyset} \cdot \frac{a\emptyset}{C_2 + a\emptyset} \qquad \text{---} \quad (3.1)$$

and

$$\underset{2 \rightarrow 1}{\text{Transfer}} = V_2 \frac{C_2}{C_2 + a\phi} \cdot \frac{\phi}{C_1 + \phi}$$

where V_1 and V_2 are rate constants. However when the concentrations C_1 and C_2 are equal, there is no net transfer. This require that $V_2 = aV_1$.

Another suggestion of this hypothesis is that exchange of sugar molecules between the two opposing sites can occur if they are both occupied, but there would be no net movement of sugar. However, the net transfer may be represented by:

$$\underset{1 \rightarrow 2}{\text{Net transfer}} = \frac{V_1 C_1 . a\phi + V_{EX} C_1 C_2 - V_1 C_2 a\phi - V_{EX} C_1 C_2}{(C_1 + \phi)(C_2 + a\phi)}$$

where V_{EX} is the exchange rate constant. —— (3.2)

In the presence of a competitive inhibitor, one has to take into consideration the concentration and the half-saturation constant for that inhibitor, thus the saturation fraction in the presence of a competitive inhibitor takes the form:

$$\frac{C}{C + \phi(1 + I/\phi_I)}$$

where I and ϕ_I are the inhibitor concentration and the half-saturation constant for the inhibitor, respectively. Therefore, under competition, equation 3.2 takes the following form:

$$\underset{1 \rightarrow 2}{\text{Net transfer}} = \frac{V_1 a\phi(C_1 - C_2) + V_{EX}(C_1 C_2 - C_2 C_1)}{[C_1 + \phi(1 + I_1/\phi_I)][C_2 + a\phi(1 + I_2/i\phi_I)]}$$

—— (3.3)

where i is the asymmetry factor for the affinity of the inhibitor for the system.

Consequently, for the Sen-Widdas exits procedure, which essentially entails the exits of sugar from preloaded red cells into media containing very low sugar concentrations and/or low inhibitor concentrations, the equation 3.3 can be simplified by neglecting C_1 in the numerator and also the exchange term to give:

$$\text{Exit rate} = \frac{V_1 a \phi C_2}{[C_1 + \phi(1 + I_1/\phi_I)][C_2 + a\phi(1 + I_2/i\phi_I)]} \quad \text{—— (3.4)}$$

In practive, however, the exit time, which is proportional to the reciprocal of the exit rate is used. The extrapolation of the linear part of the exits record to the equilibrium level gives the exit time. Therefore:

$$\text{Exit time} = \frac{S'}{V_1 a \phi}\left[C_1 + \phi\left(1 + \frac{I_1}{\phi_I}\right)\right]\frac{[C_2 + a\phi(1 + I/i\phi_I)]}{C_2} \quad \text{—— (3.5)}$$

where S' is the initial sugar concentration.

Thus the exit time is shown to be a linear function of the outside sugar concentration in the absence of any inhibitor, or in the presence of a constant concentration of inhibitor. The exit time is also a linear function of the inhibitor concentration if C_1 is held constant. Furthermore, the half-saturation constant, which is equal to that value of C_1 which doubles the exit time relative to when $C_1 = 0$, is changed when the inhibitor competes for the outside site and not the inside site. The uninhibited half-saturation constant would, therefore, be a value of C_1 equal to the outside half-saturation constant.

In summary, therefore, the Sen-Widdas exits technique is envisaged as giving the half-saturation concentration of the outside site and will only show any change in this constant if the inhibitor competes for the outside site.

These kinetics also provide an interpretation of the inhibitory effects of the equilibrium exchange procedure. To reiterate, the equilibrium exchange procedure entails measurements of unidirectional fluxes where the inside and outside sugar concentrations are equal. The half-saturation constant in this case, is determined by plotting the reciprocal of fluxes, measured at different sugar concentrations against the reciprocal of these sugar concentrations in a typical Lineweaver-Burk type plot.

The unidirectional flux is given by:

$$J = \frac{V_1 a\emptyset C + V_{EX} C^2}{[C + \emptyset(1 + I/\emptyset_I)][C + a\emptyset(1 + I_2/i\emptyset_I)]} \quad \text{—— (3.6)}$$

The V_{EX} term is larger than V_1 and as C increases the V_1 term may be neglected, thus:

$$\frac{1}{J} = \frac{1}{V_{EX}}\left[1 + \frac{\emptyset}{C}\left(1 + \frac{I_1}{\emptyset_I}\right)\right]\left[1 + \frac{a\emptyset}{C}\left(1 + \frac{I_2}{i\emptyset_I}\right)\right] \quad \text{—— (3.7)}$$

The equilibrium exchange flux in the absence of any inhibitor can be described empirically by a simple relationship of the form:

$$J = V_{EX} \cdot \frac{C}{C + \emptyset_{EX}} \quad \text{—— (3.8)}$$

or

$$\frac{C}{J} = \frac{1}{V_{EX}} \cdot (C + \emptyset_{EX}) \quad \text{—— (3.9)}$$

With a simple competitive inhibitor, the reciprocal of the flux takes the form:

$$\frac{1}{J} = \frac{1}{V_{EX}}\left[1 + \frac{\emptyset}{C}\left(1 + \frac{I}{\emptyset_I}\right)\right] \qquad \text{——(3.10)}$$

and for a non-competitive inhibitor

$$\frac{1}{J} = \frac{1}{V_{EX}}\left[1 + \frac{\emptyset}{C} + \frac{I}{\emptyset_I}\left(1 + \frac{\emptyset}{C}\right)\right] \qquad \text{——(3.11)}$$

Equation 3.9 is in the Hanes plot form (Hanes, 1932), where the concentration multiplied by the reciprocal of the flux is a linear function of the concentration and the intercept on the abscissa is equal to the half-saturation concentration and the intercept on the ordinate is equal to $\emptyset_{EX}/V_{EX}$.

In summary, therefore, these kinetics describe equilibrium exchange which in the absence of any inhibitor give the half-saturation constant for exchange. It has been shown (Baker & Widdas, 1973b,Equation 18) that the exchange half-saturation constant approaches that of the higher valued site (that is the inside site for human red cells with common sugars).

In conclusion, a measure of the asymmetry factor can be determined from a combination of these procedures (Widdas, 1974). The Sen-Widdas exits technique will give an indication of the affinity and reactiveness of the outside site, whilst the equilibrium exchanges will give an approximation of the affinity of the inside site for the transport of sugars.

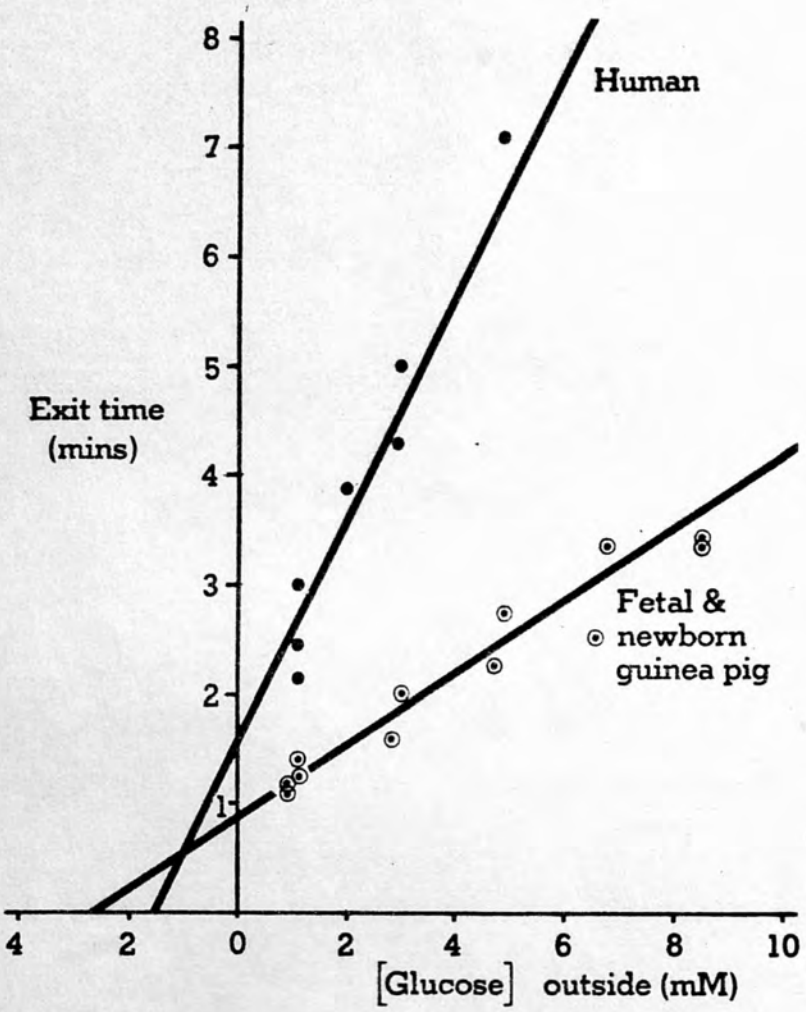


Figure 3.1

The effect of increasing glucose concentration on the exit time of 76 mM glucose from fetal and newborn guinea-pig and human erythrocytes, at 16 ^{o}C. The Sen-Widdas half-saturation constant is equal to the intercept on the abscissa.

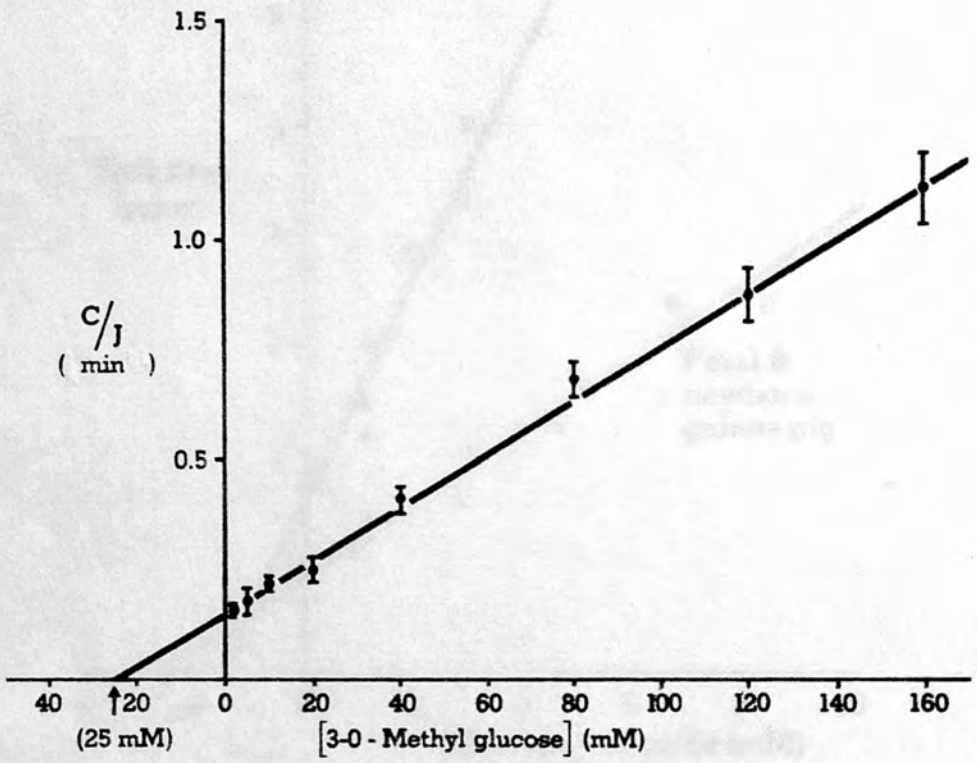


Figure 3.2

Hanes plot of 3-O-methyl glucose exchanges in the range 2-160 mM in fetal and newborn guinea-pig erythrocytes, at 16 ^{o}C. The intercept on the abscissa represents the half-saturation concentration for exchange. Points represent mean and standard deviation of 3 results.

Results

Glucose exits were measured from both fetal and newborn guinea-pig and human red blood cells and the data treated as described earlier.

The Sen-Widdas half-saturation concentration for fetal and newborn guinea-pig red blood cells was estimated to be ca. 2.7 mM at 16 ^{o}C and pH 7.4. The half-saturation concentration for human cells was found to be ca. 1.5 mM under identical conditions. The maximal transfer rates, K (see Sen and Widdas, 1962a) for newborn guinea-pig and human cells was estimated to be 0.26 and 0.15 isotonic units min^{-1}, respectively. A Sen-Widdas plot of exits at 16 ^{o}C is given in Figure 3.1.

The equilibrium exchange fluxes of 3-O-methyl glucose were measured in the range 2-160 mM at 16 ^{o}C, pH 7.4 for fetal and newborn guinea-pig red blood cells. A Hanes plot of these results is given in Figure 3.2.

The exchange half-saturation concentration for fetal and newborn guinea-pig blood was found to be ca. 25 mM and V_{EX} ca. 167 m mole ℓ^{-1} min^{-1}. The corresponding half-saturation concentration (ca. 15 mM) for human cells was less than that for newborn guinea-pig cells. Similarly the V_{EX} (ca. 119 m mole ℓ^{-1} min^{-1}) was also less for human cells.

In agreement with the findings of Dawson and Widdas (1964), the fetal and newborn guinea-pig red cells have been found to possess a faster rate of hexose transfer than human cells,

Table IIIi

The half-saturation constants and the maximal transfer constants for both fetal and newborn guinea-pig and human red blood cells, as measured by (i) Sen-Widdas exits of glucose and (ii) equilibrium exchanges of 3-O-methyl glucose, at 16 ^{o}C and pH 7.4.

(i) Sen-Widdas exits

Blood	$\emptyset$ (mM)	V_{max} (m-mole ℓ^{-1} min^{-1})
Newborn guinea-pig	2.7	88
Human	1.5	51

(ii) Equilibrium exchange

Blood	$\emptyset_{EX}$ (mM)	V_{EX} (m mole ℓ^{-1} min^{-1})
Newborn guinea-pig	25	167
Human*	15	119

* Data from Basketter and Widdas (1978)

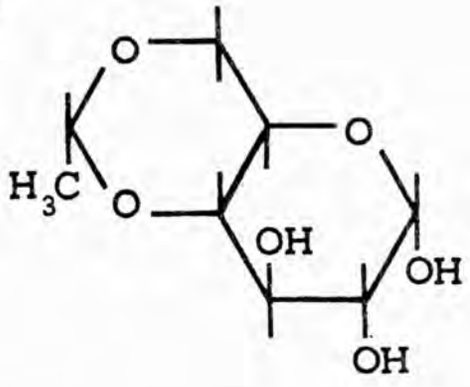
as measured by the Sen and Widdas exits procedure. In the present study, it has also been found that this is true for the hexose exchange (Table IIIi).

CHAPTER 4

THE EFFECT OF NON-TRANSPORTABLE INHIBITORS ON HEXOSE EXCHANGE

Results

(A)



(B)

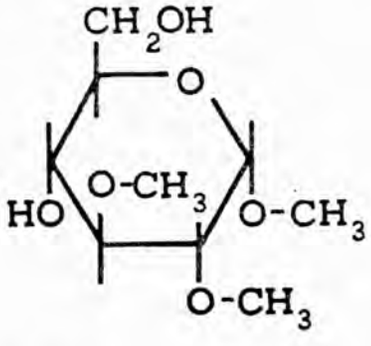


Figure 4.1

Structure of non-transportable inhibitors 4,6-O-ethylidene-α-D-glucopyranose (A) and methyl-2,3-di-O-methyl-α-D-glucopyranoside (B).

The Effect of Non-transportable Inhibitors on Hexose Exchange

The need to propose asymmetry in the hexose transport system from erythrocytes has already been discussed, however the mode of action of the non-transportable inhibitors, 4,6-O-ethylidene-α-D-glucopyranose (ethylidene glucose) and methyl-2,3-di-O-methyl-α-D-glucopyranoside (trimethylglucoside) (Figure 4.1), which provided direct evidence for asymmetry in human cells shall be discussed here.

The glucose derivative, ethylidene glucose, inhibits the Sen-Widdas glucose exits competitively, with a $K_I \simeq 5$ mM at 37 ^{o}C and pH 7.4. However, there is no mutual inhibition of ethylidene glucose penetration by glucose which would be expected of a sugar analogue with similar affinity for the hexose carrier molecule to glucose (Baker and Widdas, 1973a). One possible explanation might be that ethylidene glucose might exert its inhibitory action by binding to an adjacent site to the glucose binding site, however the finding that the temperature dependancy of the Sen-Widdas half-saturation constant for glucose and ethylidene glucose is the same as with other glucose derivatives, suggests that one membrane component for transfer must be involved (Baker and Widdas, 1973a).

Although ethylidene glucose reacts with the hexose transfer site, Baker and Widdas (1973a) have put forward evidence to suggest that ethylidene glucose does not utilize this transfer system for penetration into red blood cells, but penetrates by simple diffusion.

The rate of penetration of ethylidene glucose into red cells was found to be unaffected by the presence of either glucose or any of the inhibitors of the hexose transfer mechanisms, such as FDNB and phloretin. Further support for the mode of penetration of ethylidene glucose came from their finding that ethylidene glucose did not promote the FDNB reaction with the cell membrane, but protected the system from attack by this inhibitor (Baker and Widdas, 1973a). Krupka (1971a, b) showed that all transportable sugars accelerated the inhibition of the hexose transfer system by FDNB in a specific manner related to the affinity of these sugars for the transporting site. At the same time, he showed that the non-transported sugar, maltose, which binds to the hexose binding site on the membrane without being transported (Chen and LeFevre, 1965; Lacko and Burger , 1962) actually protects the transfer system from attack. Baker and Widdas (1973a) also found that ethylidene glucose is incapable of inducing uphill transfer by counter flow, when inside the cells, whereas other sugars with a similar Sen-Widdas K_I are able to induce this uphill transfer.

Thus this unique ability to bind with the hexose transfer system yet not utilizing it for transport and also the ability to penetrate the red cell membrane via an independent route, albeit slowly, enables one to use ethylidene glucose to make direct comparisons between the inward facing and the outward facing transfer sites, in terms of competition.

Hence, from the properties of ethylidene glucose, described above, it may be apparent that to study the inhibition of hexose transfer from the outside, it would only be necessary to suspend cells in media containing appropriate concentrations of ethylidene glucose and the usual time, of around 60 seconds, involved in sampling for flux measurements, would not allow appreciable amounts of the inhibitor to penetrate the cells and inhibit from the inside. Similarly, preloading the cells with ethylidene glucose and suspending them in ethylidene glucose-free media would therefore allow the inhibition of the internal sites to be studied before appreciable quantities of inhibitor could accumulate on the outside.

Baker and Widdas (1973b) reported that 20 mM glucose equilibrium exchanges were inhibited to a similar degree by 200 mM ethylidene glucose when present on the inside of the cells and 25 mM ethylidene glucose when present on the outside, thus revealing the difference in the

inhibitory potency of this inhibitor when present inside or when present outside. The analysis of their results suggested a tenfold asymmetry of affinity between the two sides of the membrane for glucose and a fortyfold asymmetry for ethylidene glucose. This figure was later revised, using purified ethylidene glucose, to a sixty-fold asymmetry for ethylidene glucose (Baker et al., 1978). The exchange inhibitory constant for ethylidene glucose, competing on the outside was ca. 11 mM whilst on the inside it was ca. 110 mM (Baker et al., 1978).

Barnett et al. (1975) studied the inhibition of the hexose transfer system by a series of 6-O-alkyl-D-galactoses and C-1 substituted β-D-glucopyranosides. In general they found that the D-galactoses substituted at the C-6 end of the molecule tended to be good inhibitors of this transport system when present on the outside. However, sugars substituted at the C-1 end of the molecule, had a tendency to be better inhibitors from the inside. On the basis of their findings, Barnett et al. (1975) proposed a model for transfer where the membrane component was seen to accept the glucose molecule from the C-1 end when presented from the outside and from the C-6 end when presented from the inside. (For further details see original paper).

Another non-transportable inhibitor, methyl-2,3-di-O-methyl-α-D-glucopyranoside was also studied by Baker et al. (1978), in terms of asymmetry of affinities between the two sides of the membrane. The trimethylglucoside molecule is bulky towards the C-1 end, thus, in agreement with

the postulated model of Barnett et al. (1975), this inhibitor shows a smaller degree of asymmetry in the transport system than does ethylidene glucose. Trimethylglucoside shows asymmetry in the reverse direction to ethylidene glucose with an apparent K_I for the inside of ca. 135 mM while that for the outside site was ca. 290 mM (16 ^{o}C).

In this work it was decided to investigate whether the hexose transport system in red blood cells from newborn guinea-pigs exhibited such asymmetry in affinities, between their internal and external transfer sites, towards the non-transportable inhibitors ethylidene glucose and trimethylglucoside.

Results

Inhibition of 20 mM 3-O-methyl glucose equilibrium exchange, from newborn guinea-pig red blood cells, was measured at 16 ^{o}C either with up to 200 mM ethylidene glucose present inside the cells or up to 50 mM ethylidene glucose present outside. With very high concentrations of ethylidene gluocose inside the cells, it was found necessary to provide osmotic compensation to minimise volume changes during measurements. This osmotic balancing was achieved by having appropriate amounts of inositol and malonamide present in the suspending media.

That the reciprocal of the exchange flux should be a linear function of inhibitor concentration is predicted

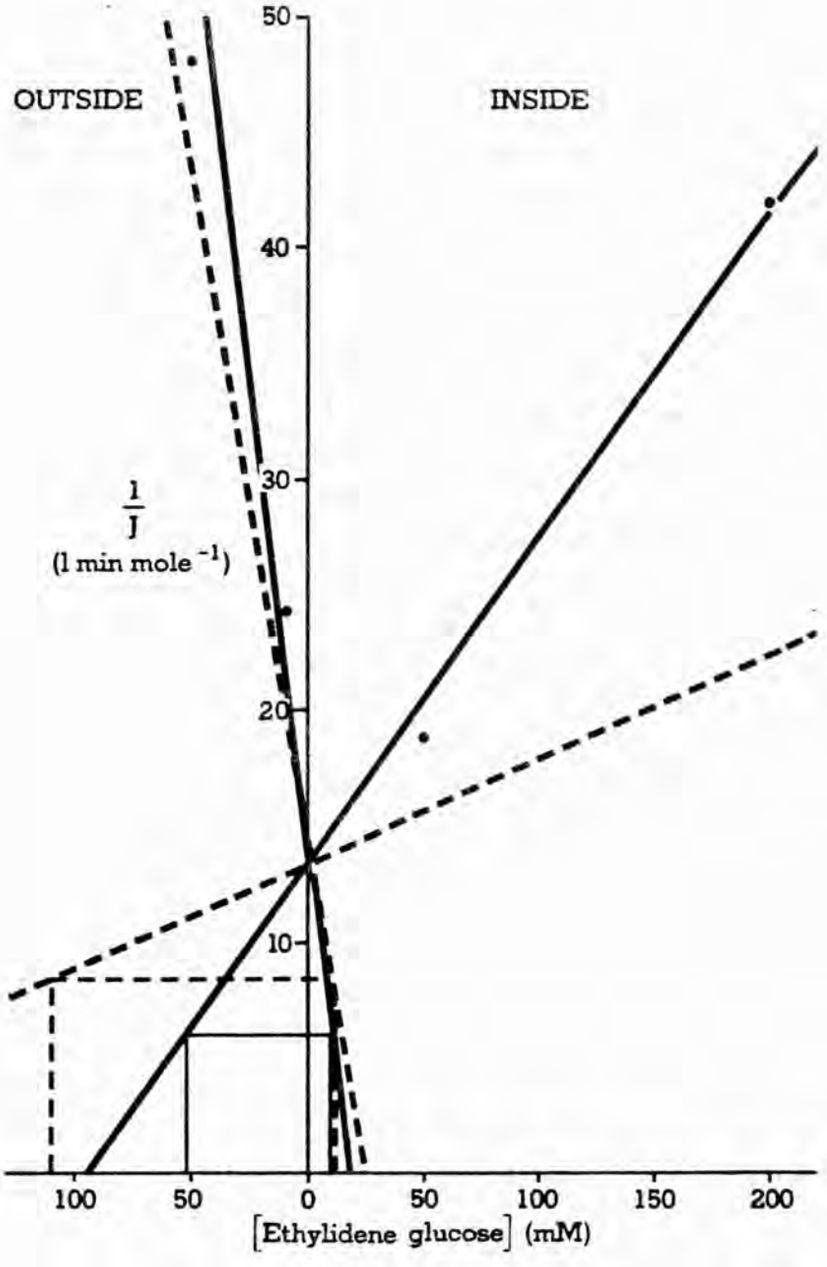


Figure 4.2

Asymmetric inhibition of 3-O-methyl glucose exchange by purified ethylidene glucose. Points are a mean of two similar results for 20 mM 3-O-methyl glucose exchange in the newborn guinea-pig cells at 16 ^{o}C. The interrupted lines represent the corresponding results for human cells, obtained by Baker et al. (1978). The intercept of the lines with a line through respective $1/V_{EX}$ parallel to the abscissa corresponds to the half-saturation concentrations for the inhibitor.

by kinetics developed by Baker et al. (1978). The intercept of a line so obtained with a line through $1/V_{EX}$, parallel to the abscissa, corresponds to the half-saturation concentration of the inhibitor. Figure 4.2. represents the data for inhibition of 3-O-methyl glucose exchange by internal and external ethylidene glucose.

A marked asymmetry in inhibition is observed with ethylidene glucose when present on the two sides respectively. More ethylidene glucose was required to produce inhibition from inside, compared with similar inhibition from the outside. The results in Figure 4.2 suggest a K_I of ca. 52 mM for ethylidene glucose when acting on the inside of the cells and a K_I of ca. 10 mM when acting outside, thus exhibiting a fivefold asymmetry in the apparent affinity for ethylidene glucose towards the hexose transfer system present in newborn guinea-pig red blood cells. This is in contrast to a tenfold asymmetry found in human cells.

It should be noted that the intercept on the ordinate is similar for both newborn guinea-pig and human cells. This is so, because at 20 mM 3-O-methyl glucose, the saturation fraction for human cells is greater than for newborn guinea-pig cells and this partly compensates for the lower V_{EX} in human cells.

The inhibition of the hexose transfer system by trimethylglucoside was similarly studied, however the newborn guinea-pig cells seem to be more fragile than human cells in the sense that while loading the cells with 200 mM trimethylglucoside, it was found that the newborn guinea-pig

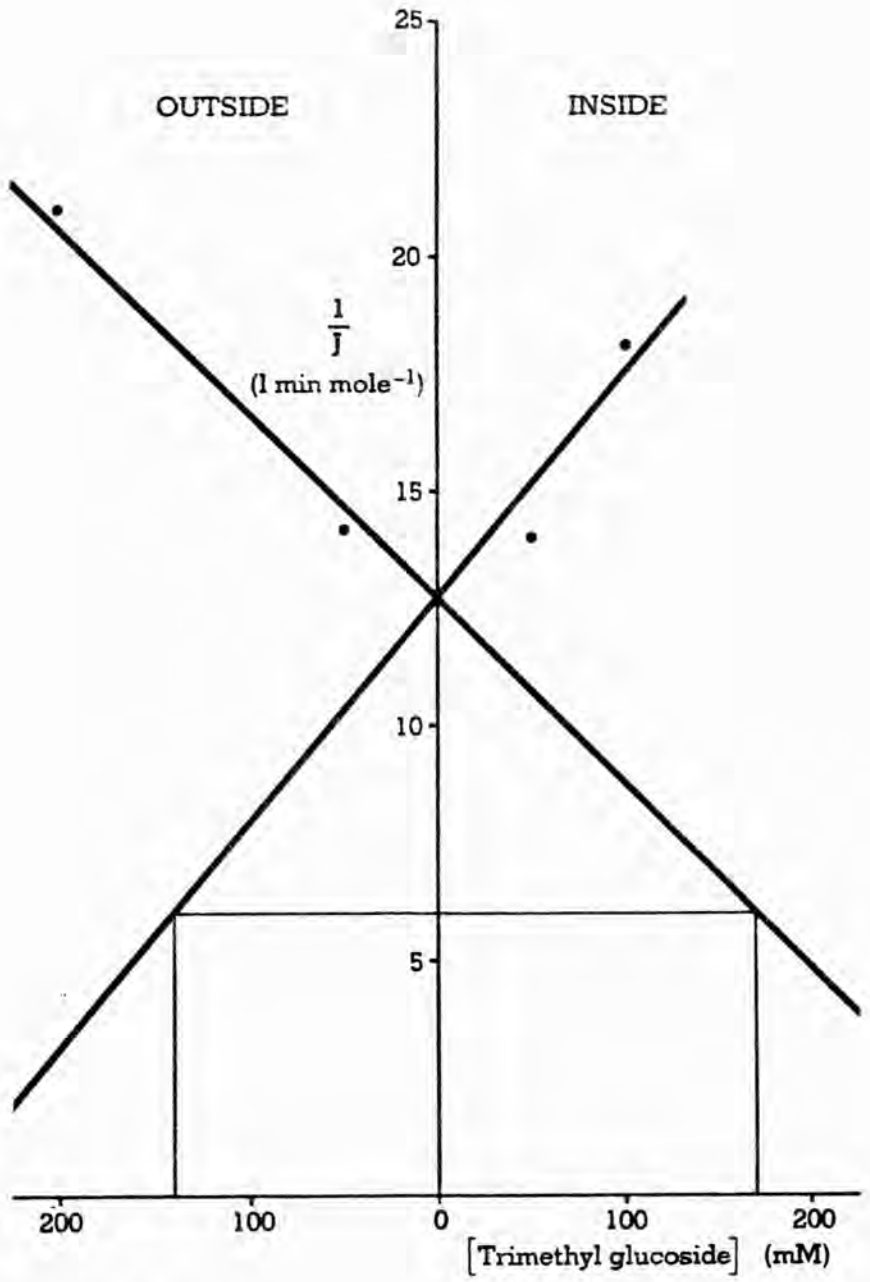


Figure 4.3

Asymmetric inhibition of 20 mM 3-O-methyl glucose at 16 °C by trimethyl glucoside in fetal and newborn guinea-pig erythrocytes. Points are means from two experiments with similar results. The line parallel to the abscissa is through $1/J = 1/V_{EX}$ and the two intercepts correspond to values of trimethyl glucoside concentration equal to the K_I for the inside and the outside sites, respectively.

Table IVi

The exchange inhibitory constants (K_I) for the inside and outside hexose transfer sites, from newborn guinea-pig and human cells for the two non-transportable inhibitors at 16 °C.

Inhibitor	Blood	Inside K_I (mM)	Outside K_I (mM)
Ethylidene glucose	N-b guinea-pig	52	10
	Human*	110	11
Trimethyl glucoside	N-b guinea-pig	140	170
	Human*	135	290

* Data for human blood obtained from Baker et al. (1978)

cells hemolysed. It was found that these cells could be satisfactorily loaded with 100 mM trimethylglucoside with only slight hemolysis.

The trimethylglucoside results (Figure 4.3) show that this derivative is able to inhibit 20 mM 3-O-methyl glucose exchange in newborn guinea-pig cells more effectively when present inside the cells than when present outside, thus displaying an asymmetry in the reverse direction to ethylidene glucose. Trimethylglucoside gave similar results with human cells (Baker et al., 1978).

In conclusion, therefore, the fivefold asymmetry shown by the hexose transfer system from newborn guinea-pig cells towards ethylidene glucose is in contrast to a tenfold asymmetry in human cells, but with lower affinities for the inward facing side in both cases. Trimethylglucoside shows similar asymmetry in both newborn guinea-pig and human cells, however it is in the reverse direction to ethylidene glucose. It would thus seem that newborn guinea-pig and human red blood cells possess a hexose transfer system which reacts in an almost identical manner, particularly with respect to the asymmetry of affinities between the inward and the outward facing transport sites for non-transportable inhibitors (Table IVi).

CHAPTER 5

INHIBITION AND BINDING STUDIES OF HEXOSE TRANSFER SYSTEM IN FETAL AND NEWBORN GUINEA-PIG ERYTHROCYTES WITH CYTOCHALASIN B

Results

1. Sen-Widdas exits

2. Equilibrium exchanges

3. Cytochalasin B binding

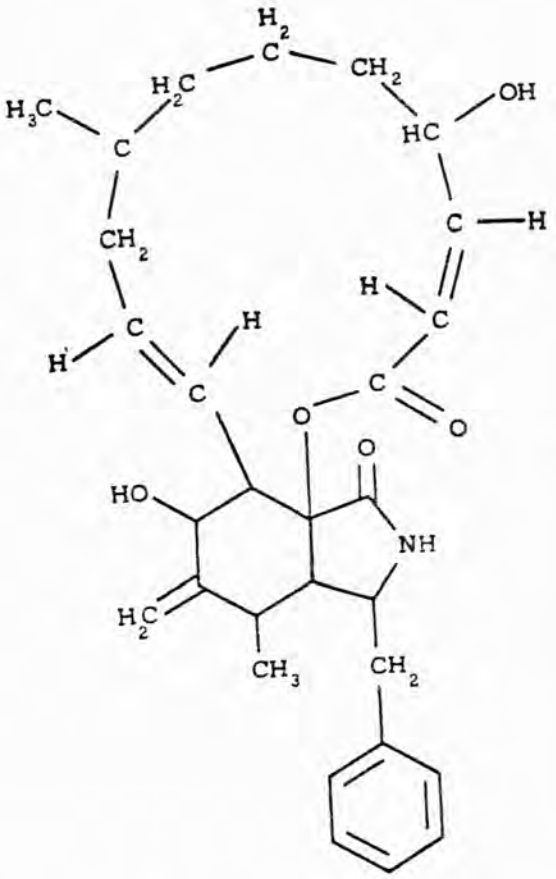


Figure 5.1
Molecular structure of Cytochalasin B.

Inhibition and Binding Studies of Hexose Transfer Sytem in Fetal and Newborn Guinea-pig Erythrocytes with Cytochalasin B

Cytochalasin B, a fungal metabolite, has a novel macrolide structure, in which the lactone ring is joined to a bicyclic lactam system (Figure 5.1).

The Cytochalasin family of compounds has been shown to have a wide range of effects, on an equally wide range of cells. This diverse range of actions includes effects on cellular movement (Wessells et al., 1971), platelet aggregation (Shepro et al., 1970), clot retraction (Majno et al., 1972), thyroid secretion (Williams and Wolff, 1971) and cytoplasmic streaming (Thomas et al., 1974). It exerts an inhibitory effect on phagocytosis (Allison et al., 1971). An inhibitory action has also been shown to be exerted on the hexose transport system in many cells (Kletzien et al., 1972; Kletzien and Purdue, 1974; Mizel and Wilson, 1972).

Cytochalasin B, in its mode of action as an inhibitor of the hexose transfer system, has been used by many workers in the field to study its various effects.

Taverna and Langdon (1973a) found Cytochalasin B to be a powerful non-competitive inhibitor of the glucose transfer system in human cells, which had a kinetically determined K_i of ca. 0.70 μM, when measured by an optical method (Sen and Widdas, 1962a, b) and 0.34 μM when

measured by the O_2 uptake procedure (Taverna and Langdon, 1973b). They also carried out equilibrium binding studies which revealed that there were 3.3×10^5 Cytochalasin B binding sites per erythrocyte with a dissociation constant of 40 nM.

The study of inhibition of the hexose transport system in the human erythrocytes by Cytochalasin B was also undertaken by Bloch (1973). Bloch, similarly, concluded Cytochalasin B to be a very potent non-competitive inhibitor of the hexose transfer system, with K_i of 5×10^{-7} M, and that at all inhibitor concentrations, the effect is readily reversible by washing the cells in inhibitor-free solutions.

Lin and Spudich (1974) investigated the binding of Cytochalasin B with the human red blood cells. Analysis of their binding studies showed that Cytochalasin B rapidly and reversibly binds to human cells, and that there are two classes of binding sites (i) a high affinity binding site (dissociation constant $\simeq 10^{-7}$ M; 3×10^5 sites per cell) and (ii) a low affinity binding site (dissociation constant $\geqslant 10^{-3}$ M). The high affinity binding sites in the human erythrocyte are membrane-associated and considerations of competition, dissociation constants, number of sites per cell and treatment of intact cells by proteolytic enzymes suggest that at least 80% of these binding sites are intimately related to the hexose transfer system.

The effect of Cytochalasin B on the hexose transfer system in human red blood cells was re-examined by Jung and Rampal (1975) in an attempt to resolve the discrepancy between workers who found Cytochalasin B inhibition to be non-competitive (see above) and workers who reported that Cytochalasin B binding at high affinity sites in the membrane is displaced in a specific manner by D-glucose and other transport substrates, indicating the competitive nature of this binding. (Lin and Spudich, 1974; Kletzien et al., 1972; Estensen and Plagemann, 1972 and Kletzien and Purdue, 1973). The isotopic measurements of equilibrium exchanges revealed that with intact cells the inhibition was competitive at glucose concentrations between 10 and 100 mM, however inhibition was non-competitive below 10 mM. With hemoglobin-free ghosts, on the other hand, Jung and Rampal showed that Cytochalasin B inhibition was competitive regardless of sugar concentration.

Further evidence for the competitive nature of Cytochalasin B was produced by Taylor and Gagneja (1975), who showed that it inhibited glucose transfer across human erythrocyte membranes with a K_I of 1.2×10^{-7} M when measuring glucose exits by an optical method. They interpreted their finding on stereochemical grounds and suggested that a Drieding molecular model of Cytochalsin B discloses an almost identical spatial distribution of four oxygen atoms

to those implicated in the hydrogen-bonding of β-D-glucopyranose, in the C-1 conformation, to the transport protein.

Basketter and Widdas (1978) studied both glucose exits and 3-O-methyl glucose equilibrium exchanges inhibition by Cytochalasin B and combined the results of these experiments with estimations of hexose transfer-related Cytochalasin B binding. They, in agreement with other workers, concluded that Cytochalasin B was a competitive inhibitor of hexose exchange whilst apparently a non-competitive inhibitor of glucose exits. This apparent anomaly was explained by them on the basis of the hypothesis that the hexose transfer system has asymmetric affinities between the inside and the outside transfer site of a red cell, and that Cytochalasin B was seen as competing only for the internal site. This assumption is consistant with kinetics which describe asymmetry in the system (see Geck, 1971; Baker and Widdas, 1973; Regen and Tarpley, 1974 and Widdas, 1980). Basketter and Widdas (1978) view this irregularity in Cytochalsin B inhibitory property as unveiling a chemical asymmetry between the two transfer sites on opposite sides of the human erythrocyte membrane.

Additional and more direct evidence of Cytochalasin B as an internal competitor was reported by Devés and Krupka (1978b) from their zero-trans exits and entry experiments in the presence and absence of the inhibitor. Devés and Krupka (1978a, b) have shown Cytochalsin B to have a marked asymmetry of affinity between the internal and external hexose transfer sites and that it shows a high affinity for the internal site and negligible affinity for the external

site. They also point to the fact that in certain other cells like the chick embryo fibroblasts, Novikoff hepatoma cells and Hela cells, the Cytochalasin B binding sites have a reversed orientation as compared to human red blood cells, i.e. the high affinity Cytochalasin B sites are present on the external surface of the cell membrane and not the internal (Kletzien and Purdue, 1973; Estensen and Plagemann, 1972; Mizel and Wilson 1972; Plagemann and Erbe, 1975 and Mizel, 1973).

Apart from kinetic evidence for asymmetry in the Cytochalasin B interaction with red blood cell membranes, independent supportive evidence was put forward by Masiak and LeFevre (1977) and Lin and Spudich (1974). The latter authors showed that an attack by proteolytic enzymes such as trypsin and pronase on the internal surface of the cell membrane renders Cytochalasin B sensitive hexose transfer sites inactive whilst with similar attack by these enzymes on the external surface, the Cytochalasin B binding sites remain unaffected. Masiak and LeFevre (1977) showed that trypsin and chymotrypsin reduced the rate of hexose transfer when incorporated into ghosts, which were resealed, but did not affect transfer rates when in the outside medium.

In view of the present study, it is of interest to note that Cytochalasin B has been used to follow postnatal changes in rabbit erythrocyte proteins, in relation to the hexose transfer in these cells. The newborn rabbit erythrocytes possess two distinct carrier mediated hexose transfer systems (Jung et al., 1980), one of which is a high affinity Cytochalasin B-sensitive system and the other is a Cytochalasin B-insensitive system. A hexose

transfer system similar to that present in the human erythrocytes is present in fetal rabbit erythrocytes, however this rapid transfer system, which is Cytochalasin B sensitive is absent from adult rabbit erythrocytes, and only a slow, Cytochalasin B insensitive system remains. The age-related loss in the fast hexose transfer and the glucose-sensitive Cytochalasin B binding sites, which have similar time courses, suggest that these sites co-exist in the neonatal erythrocytes and are lost during postnatal development.

In conclusion, therefore, the finding that Cytochalasin B is a potent inhibitor of the hexose transfer system, acting almost exclusively on the internal surface of the erythrocyte membrane, makes this compound a very desirable tool to be used in the present study for making comparisons between the hexose transfer systems in erythrocytes from fetal and newborn guinea-pigs and humans.

Also, since Cytochalasin B has been used, many times, to estimate the number of hexose transfer sites per cell (Taverna and Langdon, 1971a; Lin and Spudich, 1974; Jung and Rampal, 1976; Basketter and Widdas, 1977), it was thought of interest that in the present study a comparison of the number of transfer sites in cells from fetal and newborn guinea-pigs should be made with those reported for humans.

Results

1. Sen-Widdas Exits

Glucose exits, as described earlier, were measured from fetal and newborn guinea-pig red blood cells, preincubated

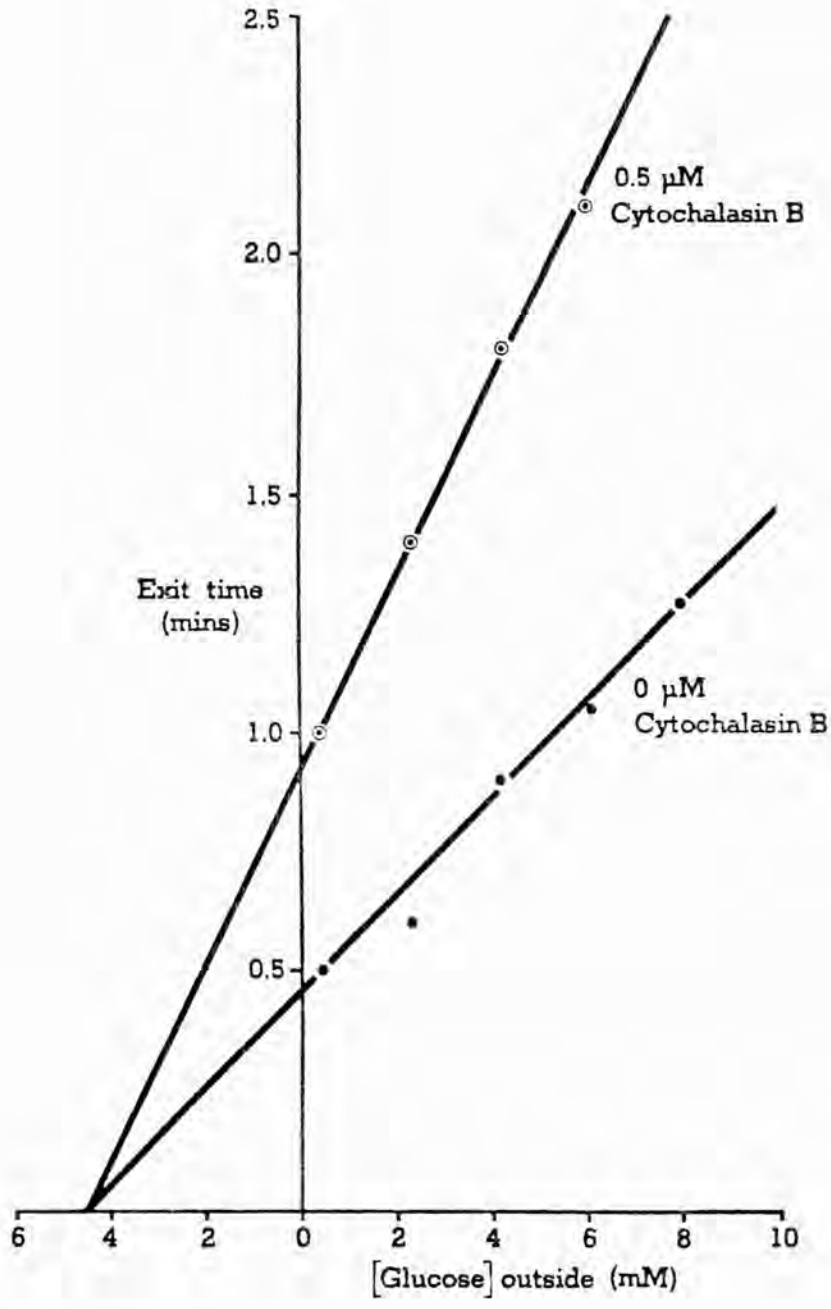


Figure 5.2

The effect of outside glucose concentration on the glucose exit time, at 27 °C, from fetal and newborn guinea-pig erythrocytes in the presence and the absence of 0.5 μM Cytochalasin B. Points • are control exits from uninhibited cells and points ⊙ are exits from inhibited cells.

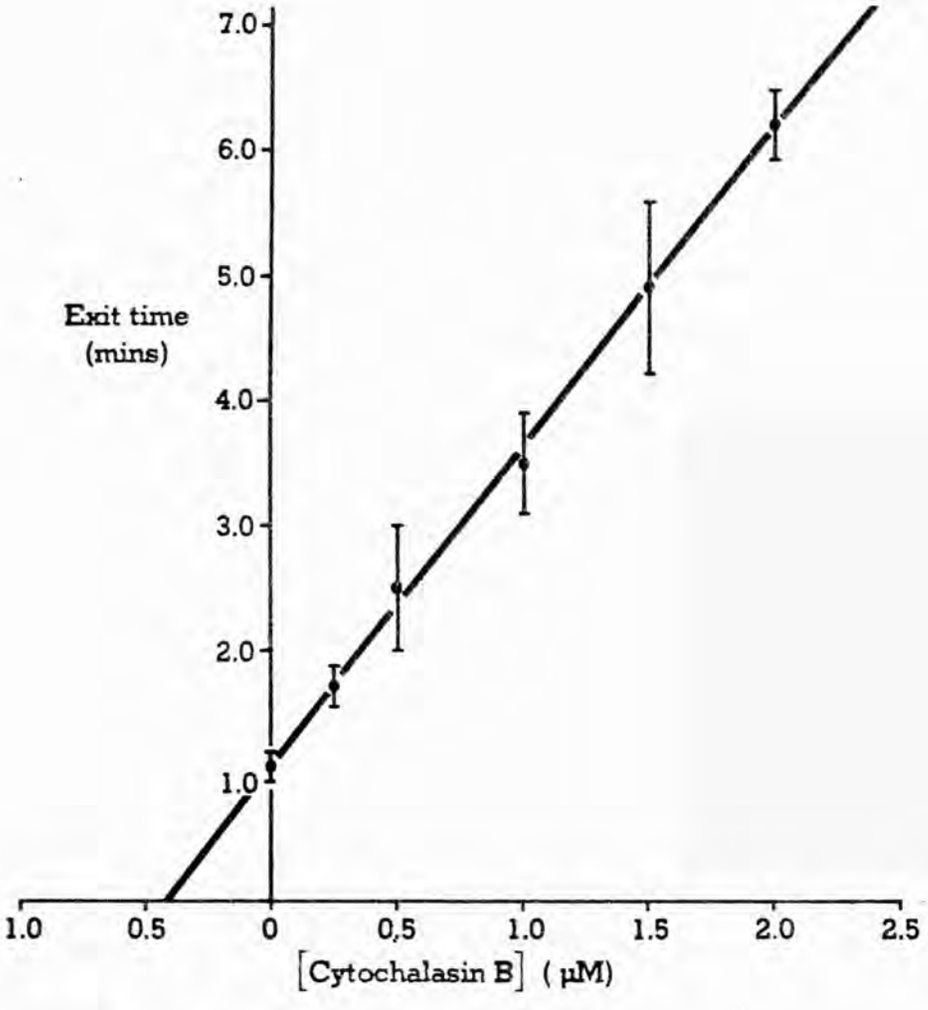


Figure 5.3

Mean and standard deviations (6 results) of exit times of 76 mM glucose into increasing concentration of Cytochalasin B, at 16 ^{o}C and pH 7.4, from fetal and newborn guinea-pig erythrocytes. The intercept on the abscissa represents the apparent inhibitory constant (K_I).

to contain 76 mM glucose into media containing various concentrations of Cytochalasin B, ranging from 0.25 to 2.0 μM. Glucose exits were also measured from these guinea-pig cells, suspended in media containing various low concentrations of glucose in the presence and absence of 0.5 μM Cytochalasin B. Glucose exits from uninhibited cells were used as controls.

Figure 5.2 shows the effect of the presence or absence of 0.5 μM Cytochalasin B on glucose exits, measured at 27 ^{o}C and pH 7.4. The intercept on the abscissa gives the half-saturation concentration for glucose, which remains unaltered in the presence of the inhibitor. The intercept on the ordinate, which is a measure of the maximal transfer rate, changes in the presence of the inhibitor, showing classical non-competitive behaviour. This apparent anomaly, however, as has been discussed earlier, is explained on the basis of an asymmetric hexose transfer system, for which the Sen-Widdas exit procedure would show a change in the half-saturation constant only if the competitive inhibitor were acting at the external site.

The apparent Sen-Widdas K_i was found to be ca. 0.42 μM at 16 ^{o}C (see Figure 5.3), which is similar to the corresponding K_i (0.5 μM) for human cells.

2. Equilibrium Exchanges

To estimate the half-saturation constant for equilibrium exchange, 3-O-methyl glucose exchange fluxes were measured in the range 2-40 mM in the presence and absence of

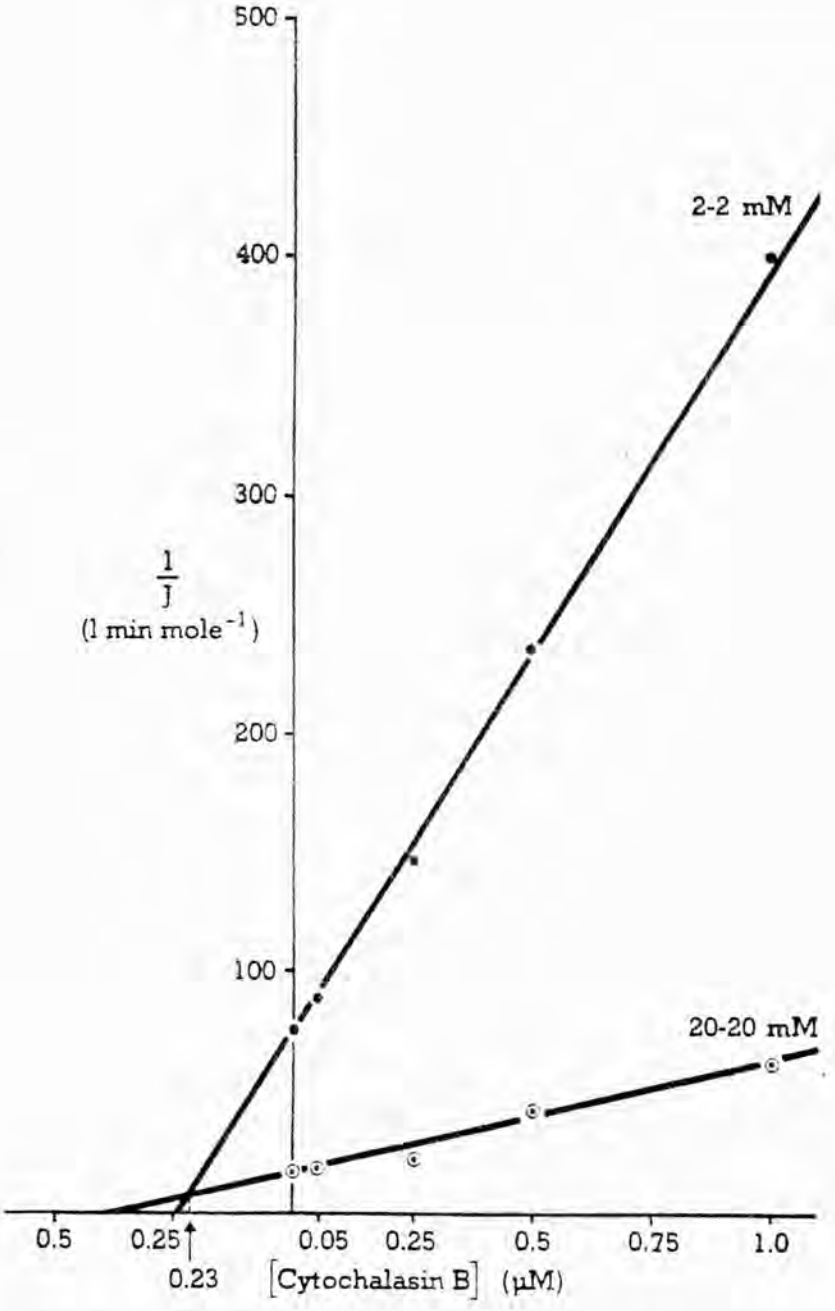


Figure 5.4

A Dixon plot for the inhibition of 3-O-methyl glucose equilibrium exchange fluxes, from newborn guinea-pig red blood cells, at 16 ^{o}C by varying concentration of Cytochalasin B. Points •, 2 mM exchange and points ⊙, 20 mM exchange. The points are a mean of two experiments with similar results.

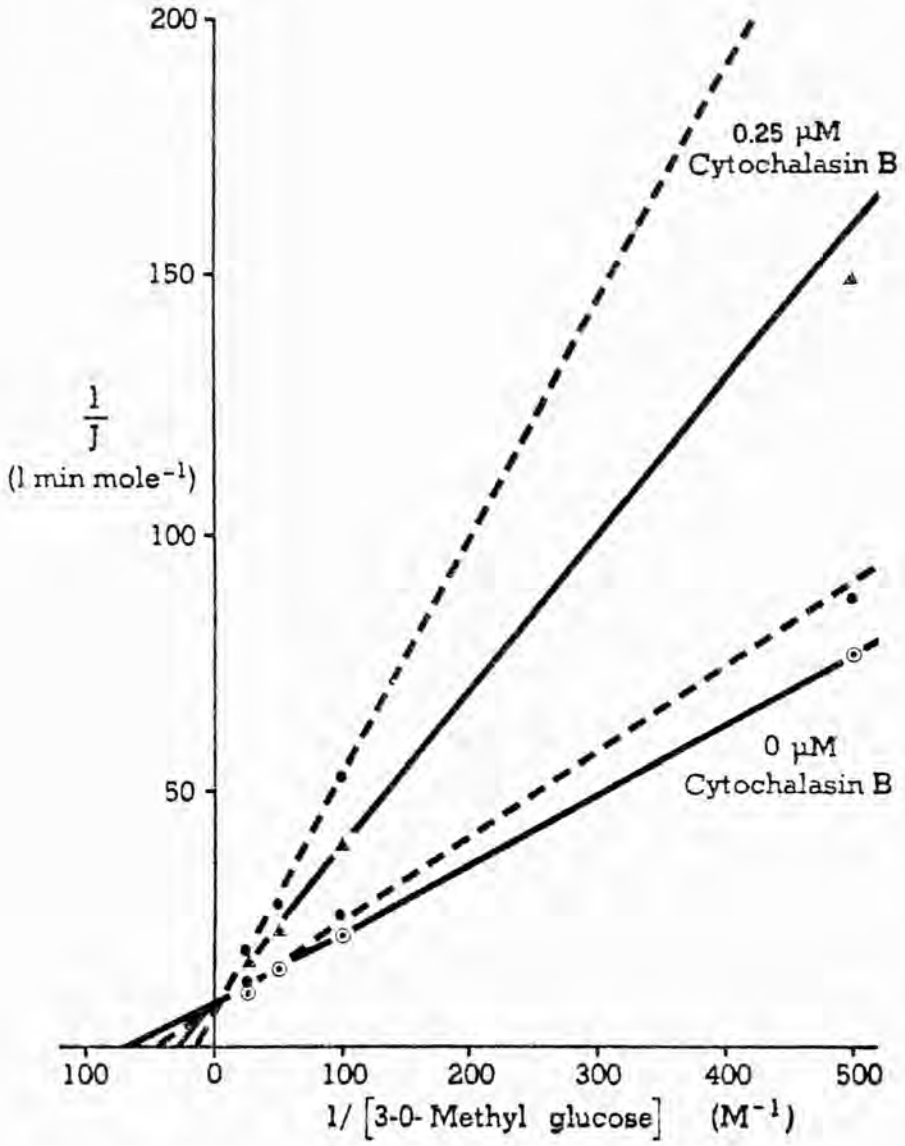


Figure 5.5

Lineweaver-Burk type plot of 3-O-methyl glucose exchange for newborn guinea-pig erythrocytes in the presence (points ▲) and in the abscence (points ⊙) of 0.25 μM Cytochalasin B. The interrupted lines represent the corresponding results for human red cells obtained by Basketter and Widdas (1978). Points are a mean of two experiments with similar results.

0.25 μM Cytochalasin B, at 16 °C for newborn guinea-pig red blood cells. The equilibrium exchanges at 2 mM and 20 mM 3-O-methyl glucose with varying concentrations of Cytochalasin B were also measured at 16 °C, pH 7.4.

The inhibition of 2 mM and 20 mM 3-O-methyl glucose equilibrium exchange at 16 °C is shown in Figure 5.4. This simple graphical method shows the inhibitory constant to be ca, 0.23 μM, which is consistent with the value calculated from the two abscissa intercepts in Figure 5.5 (ca. 0.24 μM). Figure 5.5 is a Lineweaver-Burk plot of the equilibrium exchange fluxes for newborn guinea-pig red blood cells in the presence and absence of a fixed concentration of inhibitor (0.25 μM).

The equilibrium exchange of 3-O-methyl glucose in human erythrocytes is similarly inhibited by Cytochalasin B. The K_I, measured by an identical procedure, was ca. 0.11 μM, which was almost half that for newborn guinea-pig cells.

The ratio of the K_I values for the inhibition of exchange to that for the inhibition of Sen-Widdas exits was used by Basketter and Widdas (1978) as an indicator of the site of action of particular inhibitors.

They assumed Cytochalasin B reacted only with the inside sites of the hexose transfer system and that it was

Table Vi

The inhibitory constant of Cytochalasin B, as measured by Sen-Widdas exits procedure (i) and the equilibrium exchange procedure (ii), for newborn guinea-pig cells and human cells. The data for human cells is taken from Basketter and Widdas (1978). All experiments were carried out at 16 ^{o}C, pH 7.4.

Blood	Procedure	K_I (μM)	Ratio (ii)/(i)	Site of Action
Newborn guinea-pig	(i)	0.42		
	(ii)	0.24	0.57	Mostly inside
Human	(i)	0.5		
	(ii)	0.11	0.22	Inside

characterised by a low value of this ratio (0.22 in human cells). Table Vi collects the data for newborn guinea-pig cells and human cells. The ratio for newborn guinea-pig cells is also low but is higher than that found for human cells. This is chiefly due to the higher K_I for exchange in newborn guinea-pig cells relative to that in human cells.

The inhibition of glucose exits and 3-O-methyl glucose, equilibrium exchange fluxes by Cytochalasin B has therefore shown that the fetal and newborn guinea-pig cells possess a hexose transfer system which reacts with the inhibitor in an asymmetric fashion.

The apparent non-competitive behaviour evident with glucose exits and the competitive behaviour shown by the inhibition of equilibrium exchange fluxes suggest, on the basis of kinetics adopted in the present study, that Cytochalasin B acts only or predominantly on the internal surface of the red cell membrane, in a manner which is similar to the reaction of Cytochalasin B with human erythrocytes.

3. Cytochalasin B binding

As already mentioned, in the introduction to this chapter, Cytochalasin B binds with a high affinity to red cell membranes and this binding is known to be inhibited by D-glucose and other transportable substrates (Lin and Spudich, 1974). Also the maximal saturable binding of Cytochalasin B is estimated to be between 3 and 3.3 x 10^5 molecules per cell (Taverna and Langdon, 1973; Lin et al., 1974), a value which coincides with the upper estimated value for the number of hexose transfer sites per cell in humans, that is between 1 and 3.3 x 10^5 transfer sites per cell (Masiak and LeFevre, 1972; Taverna and Langdon, 1973). Thus an intimate relationship between the saturable Cytochalasin B binding and the hexose transfer site is indicated by these findings. The displacement of Cytochalasin B by D-glucose or other transportable sugars is competitive with a one-to-one stoichiometry, thus if Cytochalasin B binding is assumed to be on a one-to-one basis with the transfer site, then an estimation of the hexose transfer related Cytochalasin B binding would represent an estimation of the number of hexose transfer sites per cell.

The uptake of Cytochalasin B by fetal and newborn guinea-pig red blood cells from cells incubated at 0.05, 0.25, 0.5 and 1.0 μM Cytochalasin B was therefore measured. The inhibition produced by these concentrations of Cytochalasin B was separately determined by measuring 2 mM and 20 mM 3-O-methyl glucose equilibrium exchange fluxes at each Cytochalasin B concentration. The

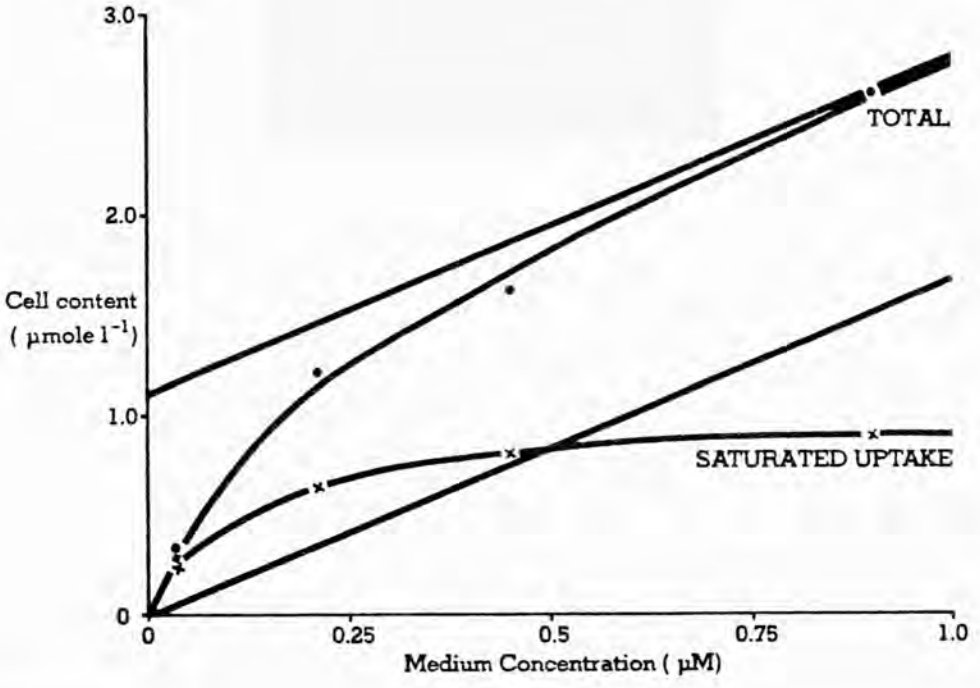


Figure 5.6

The uptake of Cytochalasin B by fetal and newborn guinea-pig erythrocytes as a function of concentration. Points • are the observed total uptake, points × represent the derived saturable uptake. The linear uptake is represented by the straight line through the origin.

Table Vii

Estimates of saturable and linear uptakes of Cytochalasin B, by newborn guinea-pig red blood cells

3-O-methyl-glucose exchange (mM)	Cytochalasin B apparent inhibitory constant (μM)	Saturable uptake X (pmoles μℓ cells^{-1})	Linear uptake Y (pmoles μℓ cells^{-1} μM^{-1})
2-2	0.248	2.05	0.88
2-2	0.248	2.37	0.74
20-20	0.414	1.38	2.79
20-20	0.414	1.52	1.95
0-0	0.23	1.84	1.25
0-0	0.23	2.42	0.28
Mean		1.93	1.31
S.E. of Mean		0.176	0.373

Cytochalasin B inhibition of the 3-O-methyl glucose fluxes provided an estimation of the apparent half-saturation concentration $\emptyset_{APP}$ (see Table Vii).

Figure 5.6 shows the uptake of Cytochalasin B by newborn guinea-pig red blood cells as a function of Cytochalasin B concentration. The figure suggests the presence of a high affinity, saturable binding component and a low affinity binding component, which is presumed to be a linear function of Cytochalasin B concentration over the range 0 to 1 μM. This is similar to the findings with human erythrocytes (Taverna and Langdon, 1973a; Basketter and Widdas, 1978).

The cellular uptake of Cytochalasin B was fitted to an equation of the following form:

$$\text{Total uptake} = X\left[\frac{I}{I + \emptyset_{APP}}\right] + YI \qquad \text{—— (5.1)}$$

where I is the Cytochalasin B concentration, X is a constant representing the saturable uptake and Y, a constant representing the linear uptake.

Values of X and Y were calculated by varying both X and Y iteratively until the sum of the square deviations for four experimentally determined points were a minimum. Six values of X and Y, respectively, were calculated. In equation 5.1 allowance was also made on the assumption that cells removed some of the Cytochalasin B from the suspending medium, for instance, when 1.0 μM Cytochalasin B was used, it was estimated that the supernatant contained only ca. 0.9 μM Cytochalasin B after incubation. Similarly 0.5 μM Cytochalasin B solution was reduced to ca. 0.45 μM,

0.25 μM to 0.21 μM and the 0.05 μM to 0.035 μM. These reduced values were used as I in equation 5.1.

Table Vii summarises the estimates of saturable and linear uptake of Cytochalasin B. The theoretical saturations have been calculated on the basis that:

$$\text{Saturation fraction} = \frac{I}{I + \emptyset_I(1 + C/\emptyset)}$$

$$\text{or} = \frac{I}{I + \emptyset_{APP}} \qquad \text{—— (5.2)}$$

where C is the sugar concentration at which inhibition was measured and $\emptyset$ the half-saturation constant for that sugar, which, for the newborn guinea-pig cells was ca. 25 mM and $\emptyset_I$ was the inhibition constant for Cytochalasin B (ca. 0.23 μM). Therefore the apparent half-saturation concentration ($\emptyset_{APP}$) at 20 mM would be 0.414 and at 2 mM it would be 0.248, while at 0 mM it would be equal to the inhibitory constant, which was put at 0.23 μM.

The mean saturable uptake, when equated with the inhibition of the hexose exchange gives a value of ca. 1.25×10^5 molecules Cytochalasin B per cell, since 1 pmole $\mu\ell$ cell^{-1} is equal to ca. 0.65×10^5 molecules/cell. On the basis of our assumption, therefore, 1.25×10^5 molecules per cell would be an estimate of the number of hexose transfer sites present in the newborn guinea-pig red cell membrane.

On the premise of equation 5.1 and the mean estimate of X and Y, saturable uptakes and the linear uptake have been plotted, also in Figure 5.6.

Table Viii

Saturable and linear Cytochalasin B binding in red blood cells from newborn guinea-pigs and humans. Also the number of hexose transfer sites per cell and the "carrier" turnover rate (at 16 ^{o}C).

Blood	Mean saturable uptake (pmoles $\mu\ell$ cells^{-1})	Mean linear uptake (pmoles $\mu\ell$ cells^{-1} μM^{-1})	Number of carrier sites/ cell	Turnover number (per second)
Newborn guinea-pig	1.93	1.31	1.25 x 10^5	1.44 x 10^3
Human*	3.98	4.18	2.4 x 10^5	0.5 x 10^3

* Data from Basketter and Widdas (1978) - who used an identical procedure for the binding studies and which were carried out in the same laboratory.

To recapitualte, the $\emptyset_{EX}$ and V_{MAX} for newborn guinea-pig cells was found to be ca. 25 mM and 167 mmoles ℓ^{-1} min^{-1} respectively and the corresponding values for humans were ca. 15 mM and 119 mmole ℓ^{-1} min^{-1}. Thus the hexose carrier in the newborn guinea-pig cells appears to have a lower affinity for 3-O-methylglucose, but possesses a higher rate of transfer.

Having estimated both the mean saturable Cytochalasin B binding and the maximal transfer rate, it is possible to calculate the turnover number for the hexose carrier. Thus the turnover number for the carriers in the newborn guinea-pig red blood cells is estimated to be 1.44×10^3 per second, at 16 ^{o}C. The turnover number for the hexose carrier in human cells, as calculated from data collected by Basketter and Widdas (1978), is 0.5×10^3 per second.

In summary, therefore, although the human cells contain almost twice as many hexose transfer sites as do the newborn guinea-pig cells, the latter appear to have a threefold faster turnover rate (Table Viii).

CHAPTER 6

INHIBITION OF HEXOSE EXITS AND EXCHANGES BY BIPHENOLIC COMPOUNDS

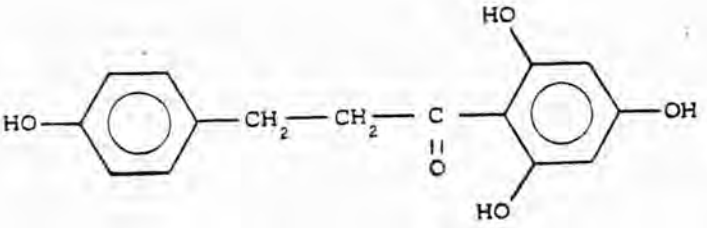
Results

1. Experiments with phloretin

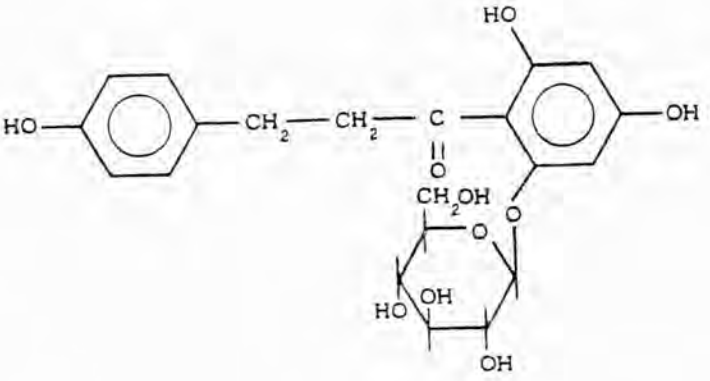
2. Experiments with phlorizin

3. Experiments with polyphloretin phosphate

(A)



(B)



(C)

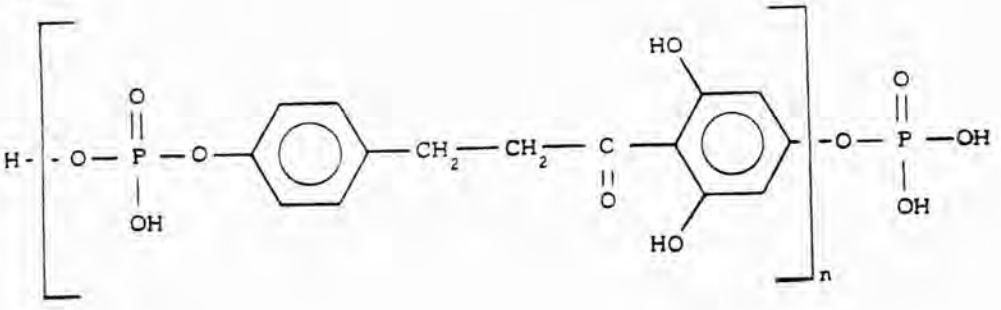


Figure 6.1

The molecular structure of phloretin (A), phlorizin (B) and polyphloretin phosphate (C). The polyphloretin phosphate chain may occur as shown, however there are a lot of branching points in the molecule (Compound information, Leo 101).

Inhibition of Hexose Exits and Exchanges by Biphenolic Compounds

Although the biphenolic compounds phloretin, phlorizin and polyphloretin phosphate have no structural analogy to sugars, they are all potent competitive inhibitors of the hexose transfer system in a number of mammalian tissues. Some of the other biphenolic compounds which belong to the same class of inhibitors, that also act on the hexose transfer system include naringenin, hexestrol, dienestrol, diethylstilbestrol and stilbestrol (LeFevre, 1959).

Historically, this group of compounds is well known for the production of glucosuria in mammals, which is induced by the inhibition of the reabsorption of glucose in the renal tubules (Poulsson, 1930).

For the present study it was desirable to use phloretin, phlorizin and polyphloretin phosphate since these compounds share a common structural unit (see Figure 6.1) and also since the most work has been done on phloretin and its related compounds. Secondly, phloretin is suggested to be able to penetrate the red cell membrane (Jennings and Solomon, 1976) whilst polyphloretin phosphate has been shown not to penetrate this membrane (Wilbrandt , 1954).

As an introduction to these compounds, a brief outline of their inhibitory action is given below.

Phloretin is a potent inhibitor of the energy independent transport processes. These, for example, include the

hexose transfer system in erythrocytes (LeFevre, 1961), fat cells (Czech et al., 1973) and rabbit heart muscle (Bihler et al., 1965). It also inhibits the ion exchange mechanism in the erythrocyte membrane (Wieth et al., 1972) and the urea transport across toad bladder (Levine et al., 1973) and erythrocytes (Macey and Farmer, 1970).

Phloretin inhibits the efflux of glucose from human erythroctyes with a K_I of 0.5-2.5 μM (LeFevre and Marshall, 1959; Sen and Widdas, 1962b; Krupka, 1971). The binding of phloretin to intact red cells and purified hemoglobin is non-saturable and is approximately equal in magnitude (Jennings and Solomon, 1976). This finding strongly suggests that most of the phloretin bound to the intact cell may be actually ascribed to hemoglobin. Jennings and Solomon (1976) further supported the contention that most of the phloretin binds to hemoglobin by showing that hemoglobin-free erythrocyte ghosts bind only 10% as much phloretin as an equivalent number of intact cells. They also found that phloretin binds at two distinct saturable sites on the hemoglobin-free ghost membranes. One of these sites is a high affinity, membrane protein related site (K_{DISS} 1.5 μM; 2.5 x 10^6 sites per cell), and the other is a low affinity site, which is confined to the lipid extract of the cell membrane (K_{DISS} 54 μM; 5.5 x 10^7 sites per cell). Thus, on the basis of these findings, it may be possible to classify the effects of phloretin, depending on the inhibitor concentrations used. Since the low affinity binding sites are half-saturated with 50 μM phloretin at pH 6 and 100 μM phloretin at pH 7.4, the phloretin effects observed at

concentrations of 100 μM and above at pH 7.4 may therefore be attributed to phloretin binding to the lipid region of the membrane (Jennings and Solomon, 1976).

Jennings and Solomon (1976) found that 100 mM glucose could not displace detectable amounts of phloretin from the erythrocyte membrane, however they concluded that this may be due to a 10% variation in their experimental results, thus 3 x 10^5 glucose inhibitable, phloretin binding sites may be present, but not detected. This apparent inability of glucose to displace the competitive inhibitor from cells, as Bowyer and Widdas (1956) have pointed out, may be due to the inhibitor not combining directly at the glucose site, but at an adjacent point where it would tend to interfere, sterically, with the glucose adsorption, thus showing competitive kinetics while not being displaced by excess glucose.

There has been some contradiction in the interpretation of data with regard to the site of action of phloretin with the sugar binding site, that is to say, some authors concluded from their work, that phloretin is unable to penetrate the red blood cell membrane, hence acted on the external surface only (Beneš et al., 1972) whilst others concluded that phloretin penetrated the cell membrane very rapidly (Jennings and Solomon, 1976; Basketter and Widdas, 1978).

Beneš et al (1972) based their conclusion of impermeability of phloretin in part from experiments on sugar influx measurements into erythrocyte ghosts and the finding that the external phloretin concentration had no

effect on this influx. They produced further supportive evidence of impermeability on the grounds that their binding and trapping studies showed phloretin to bind to the same magnitude in both intact red cells and red cell ghosts. The belief that phloretin does not penetrate red cell membrane was also based on the observation that phloretin uptake is rapid (LeFevre and Marshall, 1959; Beneš et al. 1972) and that the phloretin effect is exerted almost immediately after it is added to the cell suspensions (Wieth et al., 1974; Owen et al., 1974).

The supposition that phloretin does not penetrate the cell membrane was challenged by the finding of Taverna and Langdon (1973) that external phloretin strongly retards glucose entry into red cell ghosts and secondly, LeFevre and Marshall (1959) had earlier reported that ghosts partially depleted of hemoglobin bind much less phloretin than do intact cells. Additional support was given to this theory of penetration by Jennings and Solomon (1976) when they found that phloretin penetrated cell membranes with a permeability coefficient of 2.0×10^{-4} cm/s, when measured by the pH method. (This method is based on the fact that phloretin is a weak acid, pK ≈ 7.3, therefore if red cells are suspended in unbuffered solutions at pH near the pK of phloretin, then the extracellular pH will rise as the undissociated phloretin leaves the unbuffered extracellular medium and enters the cells). The high permeability of phloretin, they concluded, was due to the high lipid solubility of phloretin.

The exact nature of the interaction of phloretin with the red blood cell hexose transfer system was investigated by LeFevre (1959). His experiments with various molecular fragments of phloretin showed that they did not share the property of a high potency competitive inhibitor, as was phloretin, but he found that this high potency was duplicated by certain artificial estrogens resembling phloretin with respect to the spacing between the terminal phenolic hydroxyl groups. It was therefore concluded that this specially high potency of the biphenolic compounds, to inhibit the hexose transfer system, was due to their particular geometry which allows the molecules to fix at two points on the membrane matrix.

Basketter and Widdas (1978) found that the concentration of phloretin which half maximally inhibits the glucose exits from human erythrocytes was ca. 0.24 μM and that equilibrium exchange inhibitory constant was ca. 0.4 μM at 16 ^{o}C. They deduced that phloretin must react at both the inside and the outside sites, since its inhibition of exchange was much more effective than would be expected from an inhibitor acting on the external surface only.

The glucoside phlorizin, on the other hand, is a more potent inhibitor of the active transport of hexose across the kidney tubules (Chan and Lotspeich, 1962) and the small intestine (Alvarado, 1967). Specific phlorizin receptors, observed in membranes of fat cells are believed to be associated with phlorizin inhibition of hexose

transfer across these membranes (Glossmann and Neville Jr, 1972; Chesney et al., 1974).

Phlorizin inhibits the hexose transfer across human erythrocytes, competitively (LeFevre, 1954). The phlorizin concentration which half-saturates the hexose transfer system in human cells, at 37 ^{o}C, is 0.15 mM, as measured by the Sen-Widdas exit technique (Sen and Widdas, 1962b).

The glucose moiety of the phlorizin molecule does not appear to be involved in the inhibition of hexose transfer across erythrocyte membranes since the inhibition of this system is much more effective with its aglucon, phloretin (Wilbrandt, 1950). Also, when the glucose residue is substituted by a methyl group, the inhibitory power of the parent molecule is not significantly reduced (Wilbrandt and Rosenberg, 1957).

Rosenberg and Wilbrandt (1962) reported that phlorizin inhibits the hexose transfer mechanism in human erythrocytes in an asymmetric manner. Phlorizin inhibits sugar efflux while having practically no effect on its influx.

A comparative study of the effect of phloretin and phlorizin on human erythrocytes, hamster small intestine and rabbit kidney cortex slices suggested that a single type of hexose carrier molecule was present in all of these cells, however, it was also pointed out that the membrane site or sites for phloretin and phlorizin may not be identical (Kotyk et al., 1965).

It may be worthwhile to note that an interesting interpretation of their findings was made by LeFevre and Marshall

(1959) when they suggested that the apparent small activity of phlorizin could be entirely attributed to the contamination of phlorizin by phloretin and/or the hydrolysis of small amounts of phlorizin into phloretin, since only 1 to 2% hydrolysis of the phlorizin present could account for the apparent effectiveness of phlorizin. Kalckar (1936) had earlier put forward such interpretation as the basis of phlorizin's action on kidney phosphatase.

Phlorizin in hamster intestine has been shown, by a high resolution radioautographic technique, not to penetrate the cell membrane (Stirling, 1961). Phlorizin has also been shown not to penetrate the renal proximal tubule luminal membrane (Frasch et al., 1970; Glossmann and Neville Jr, 1972).

By virtue of the fact that phlorizin inhibits the active transport of hexose more effectively than phloretin, as described above, and whilst phloretin inhibits the energy independent hexose transfer almost 100 fold more effectively than phlorizin (Wilbrandt and Rosenberg, 1957) there has been a tendency for workers to use these drugs according to the preparation being studied. It is thus apparent that there is only a limited amount of literature on the action of phlorizin with the hexose transfer system in erythrocytes.

A highly polymerised form of the parent biphenol compound is polyphloretin phosphate. This polymerised form also inhibits hexose transfer across human erythrocytes

competitively (Bowyer and Widdas, 1958). Studies with ^{32}P-polyphloretin phosphate revealed that it does not penetrate the red cell membrane (Wilbrandt, 1954), thus the hexose transfer is influenced by this inhibitor via the external transfer site only. Wilbrandt (1954) reported that the efflux of glucose, from red blood cells, was inhibited while the influx of this sugar is virtually uninhibited in the presence of polyphloretin phosphate. He concluded that this difference in the inhibition of glucose exits and glucose entrance by polyphloretin phosphate was due to an asymmetry in the transport process.

Bowyer and Widdas (1958) also measured the inhibition of glucose entries and exits by polyphloretin phosphate in human erythrocytes. They found the glucose exits to be inhibited to a greater degree than glucose entry, however they accounted for this discrepancy on the basis of competition for a symmetrical system, whilst not disproving the enzyme theory of Wilbrandt (1954).

The features of phloretin, phlorizin and polyphloretin phosphate, described above, were thus anticipated to provide an opportunity to observe and compare the inhibition of the hexose transport systems in newborn guinea-pig and human red blood cells, in the hope that these observations would disclose any further differences which there may be between the transport systems in the two species.

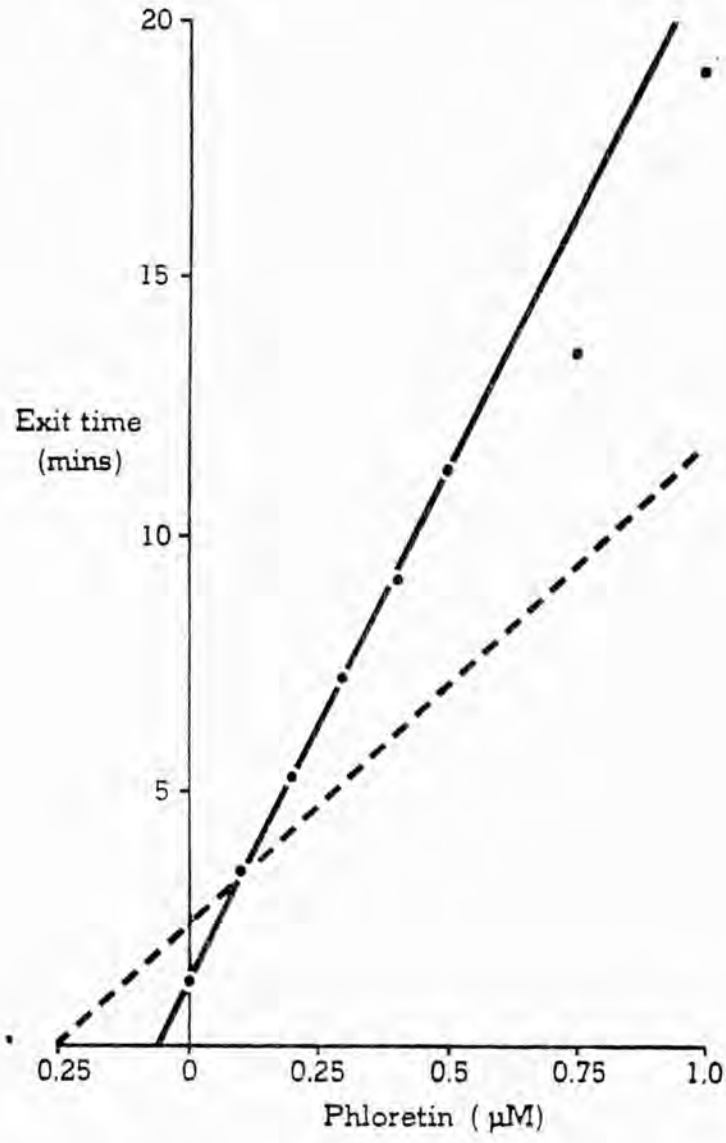


Figure 6.2

The effect of phloretin concentration on glucose exits at 16 °C from fetal and newborn guinea-pig erythrocytes preincubated to contain 76 mM glucose. The interrupted line represents the corresponding results with human cells, as obtained by Basketter and Widdas (1978). Points and continuous line are typical results.

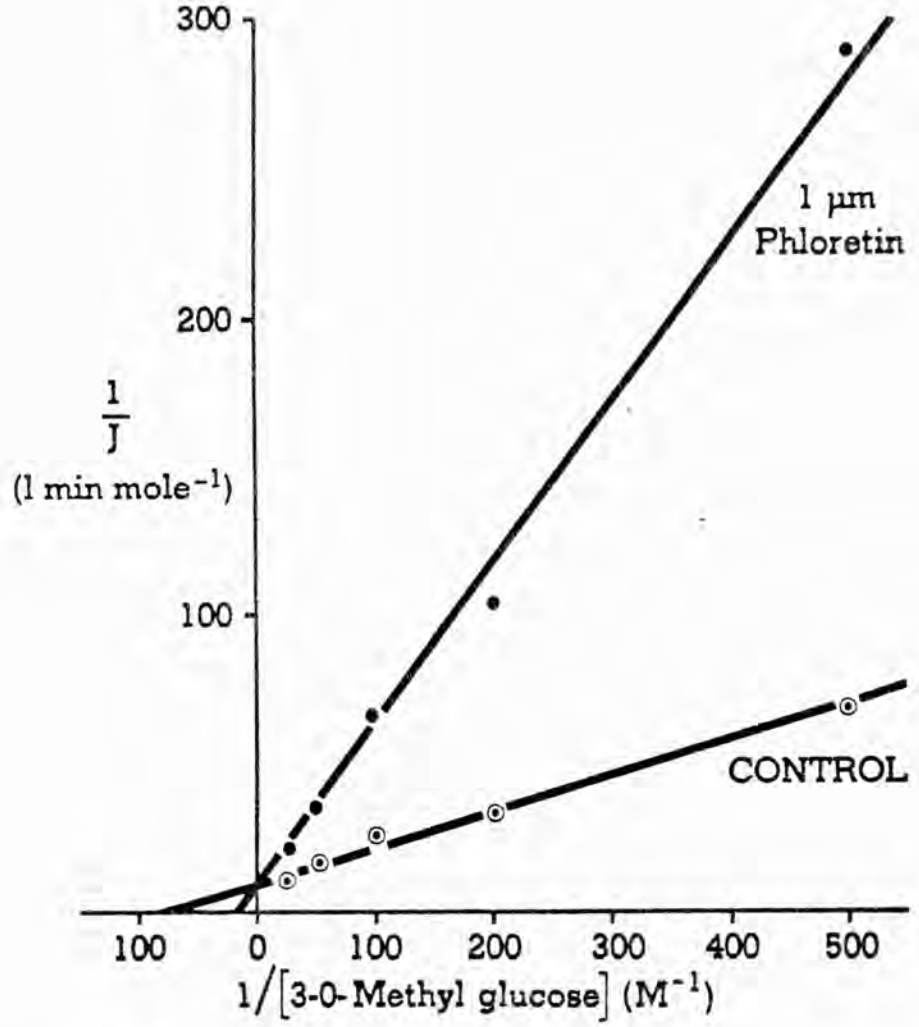


Figure 6.3

Lineweaver-Burk type plot of 3-O-methyl glucose exchange in the range 2-40 mM at 16 °C, in the presence and the absence of 1 µM phloretin. Points ⊙ are control exchanges and points • are in the presence of 1 µM phloretin. Points represent a mean of two similar experiments with fetal and newborn guinea-pig erythrocytes.

Results

1. Experiments with Phloretin

The inhibition of glucose exits, by phloretin, from newborn guinea-pigs was studied at 16 ^{o}C. The effect of phloretin concentration on glucose exits is shown in Figure 6.2. The interrupted line represents results from a corresponding experiment with human cells.

The glucose exits from newborn guinea-pig cells were more inhibited by phloretin than from human cells. The concentration which half inhibits the Sen-Widdas exits, at 16 ^{o}C, from newborn guinea-pig cells was ca. 0.068 μM compared to ca. 0.24 μM for human cells. The intercept on the ordinate, which is smaller for newborn guinea-pig cells than human cells, suggests a faster maximal exit rate in the absence of the inhibitor.

The inhibition of 2 mM and 20 mM 3-O-methyl glucose exchange by phloretin in the range 0.5 to 2.0 μM was studied at 16 ^{o}C and also the inhibition of varying concentrations of 3-O-methyl glucose, from 2 mM to 40 mM, with a constant 1 μM phloretin, was studied. A Lineweaver-Burk type plot of the results is shown in Figure 6.3. The inhibitory constant for the inhibition of 3-O-methyl-glucose exchanges with phloretin was found to be ca. 0.34 μM for newborn guinea-pig cells. This was ca. 0.4 μM for human cells at 16 ^{o}C (Basketter and Widdas, 1978). The V_{MAX} value remained unaltered in the presence of the inhibitor suggesting that inhibition was competitive in cells from both species.

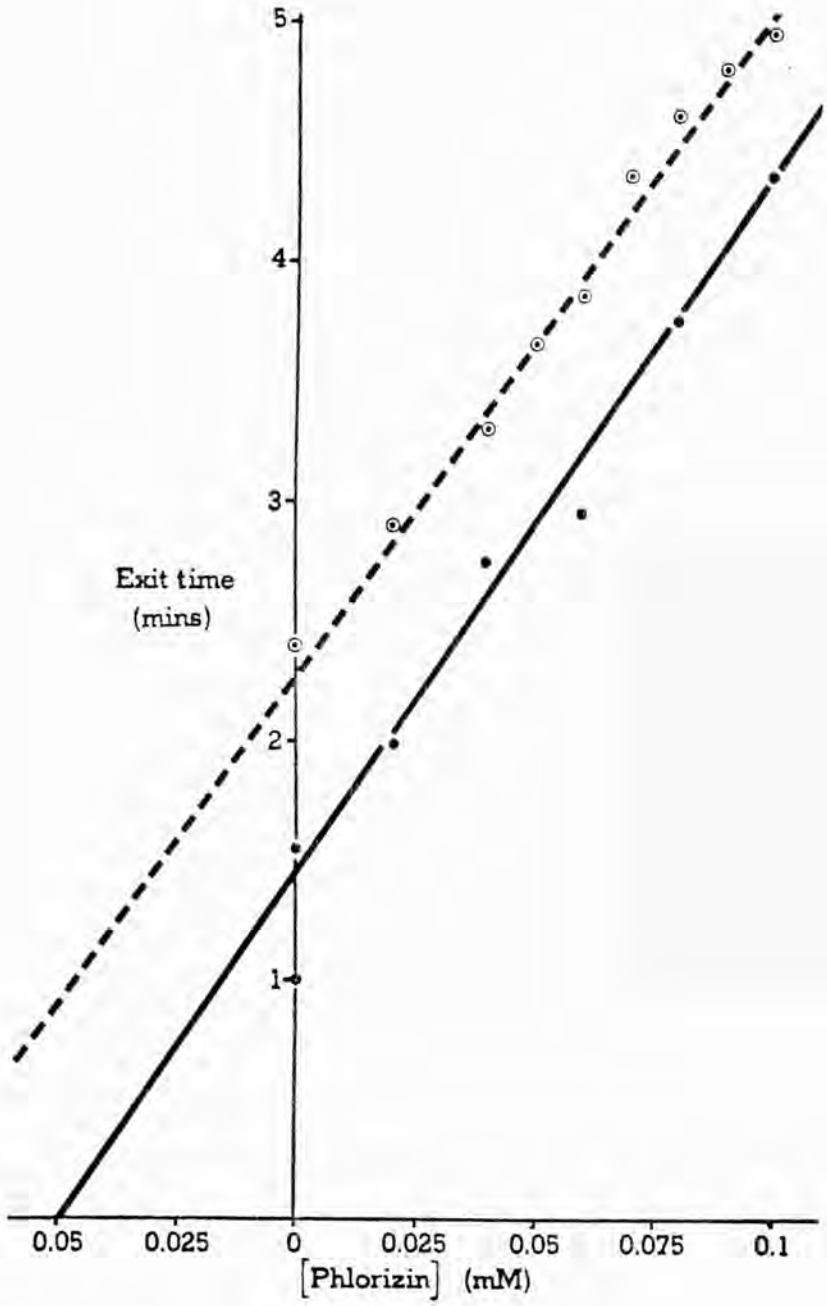


Figure 6.4

The effect of phlorizin concentration on glucose exits at 16 °C, from newborn guinea-pig red blood cells (points • and continuous line) and human cells (points ⊙ and interrupted line). The cells from both species were preincubated at 76 mM glucose and the results are typical of Sen-Widdas exit experiments with cells inhibited by phlorizin.

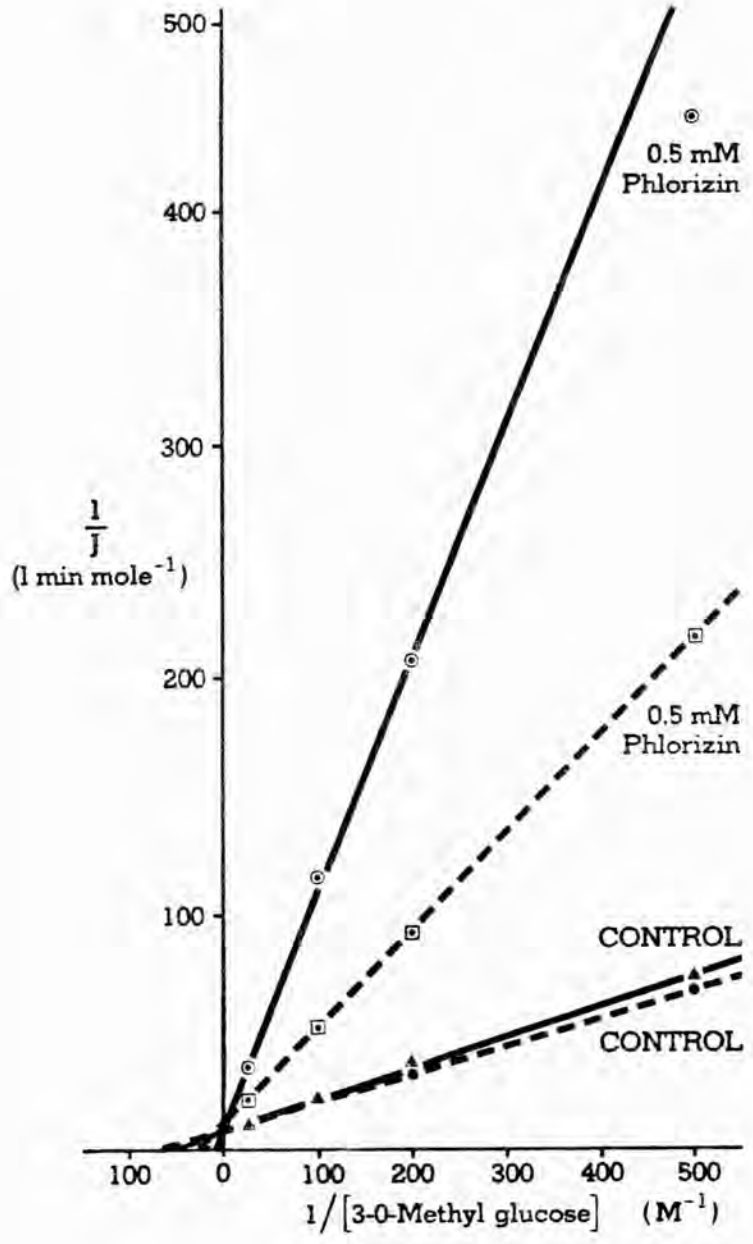


Figure 6.5

A Lineweaver-Burk type plot of 3-O-methyl glucose exchanges in the range 2-40 mM, at 16 ^{o}C, in the absence (points ● and ▲) and in the presence of 0.5 mM phlorizin (points ⊙ and ⊡). The interrupted lines are data for human erythrocytes. All points are a mean of two experiments with similar results.

Table VIi

Variations in K_I value with increasing concentrations of phlorizin, as estimated by the inhibition of 2 mM and 20 mM 3-O-methylglucose exchange at 16 °C.

Concentration of phlorizin (mM)	Apparent K_I (mM)	
	Newborn guinea-pig cells	Human cells
0.05	0.0535	0.0984
0.1	0.0574	0.1066
0.2	0.0596	0.1262
0.5	0.0655	0.1295
0 (extrapolated)	0.053	0.095

2. Experiments with Phlorizin

Glucose exits were measured from newborn guinea-pig and human red blood cells, preloaded to 76 mM, at 16 ^{o}C. The Sen-Widdas inhibitory constant was found to be ca. 50 μM and ca. 80 μM for newborn guinea-pig cells and human cells respectively. Figure 6.4 shows the inhibition of glucose exits by phlorizin at 16 ^{o}C.

The inhibition of 3-O-methyl glucose exchange by 0.5 mM phlorizin at varying concentrations of 3-O-methyl glucose in the range 2 mM to 40 mM, at 16 ^{o}C suggest that the K_I = ca. 0.08 mM for newborn guinea-pig cells and ca. 0.112 mM for human cells (see Figure 6.5). However, while studying the inhibition of 2 mM and 20 mM 3-O-methyl glucose exchange fluxes with varying concentrations of phlorizin, in the range 0.05 mM to 0.5 mM, it was noticed that the estimations of the K_I values tended to be higher at larger concentrations of phlorizin (see Table VIi). The experiments with both newborn guinea-pig cells and human cells gave similar variations of the K_I values. It was thought that this phenomena may be due to the finite time required for the phlorizin to equilibrate across the cell membrane, so that the inhibition at the higher concentrations was not so great as would have been had sufficient time for equilibration been given. However, the protocol for the experiment required the exchange flux to start as soon as the inhibitor was added.

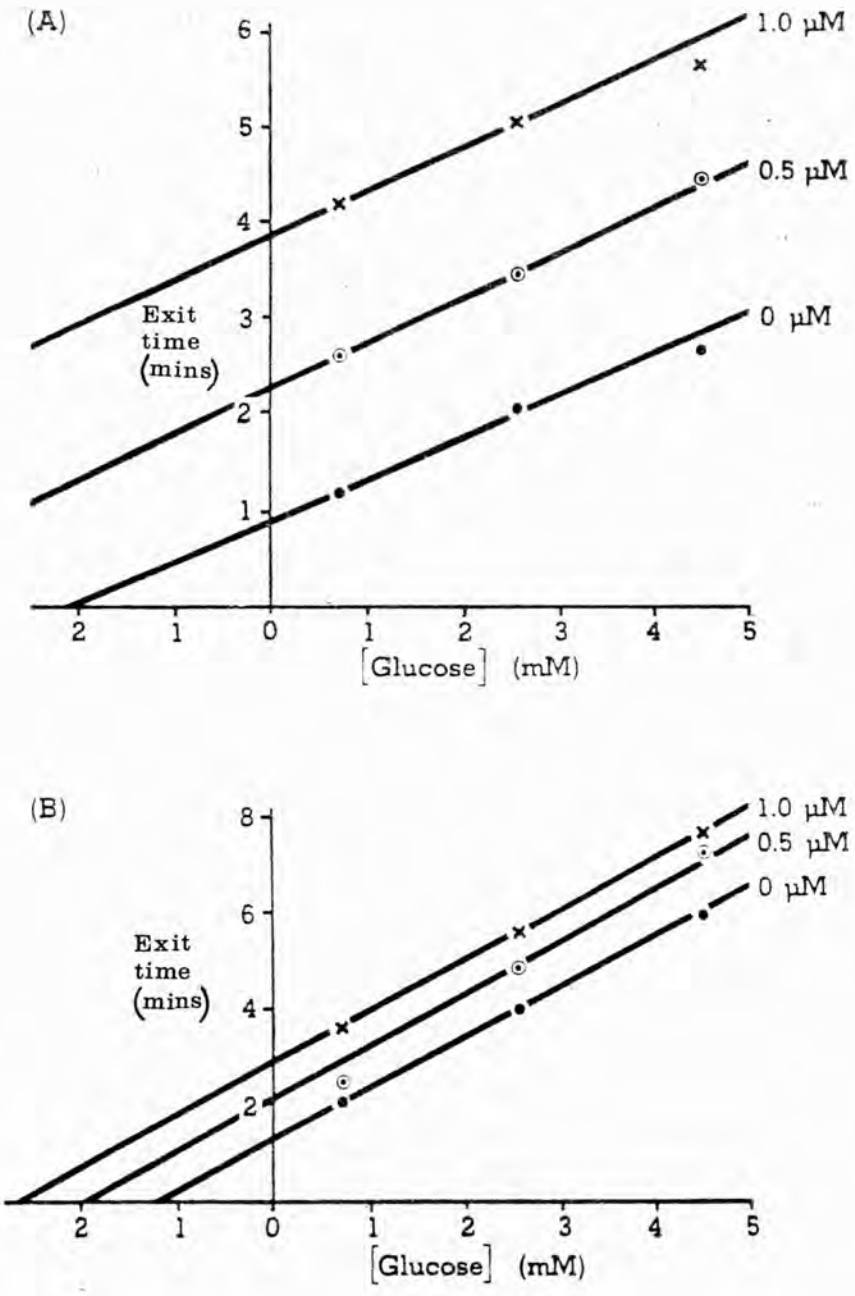


Figure 6.6

Effect of varying polyphloretin phosphate concentration on glucose exits at 16 °C, from fetal and newborn guinea-pig erythrocytes (A) and human cells (B). Points • are exits in the absence of the inhibitor, points ⊙ and × are in the presence of 0.5 µM and 1.0 µM inhibitor respectively. The results show typical competitive type of behaviour.

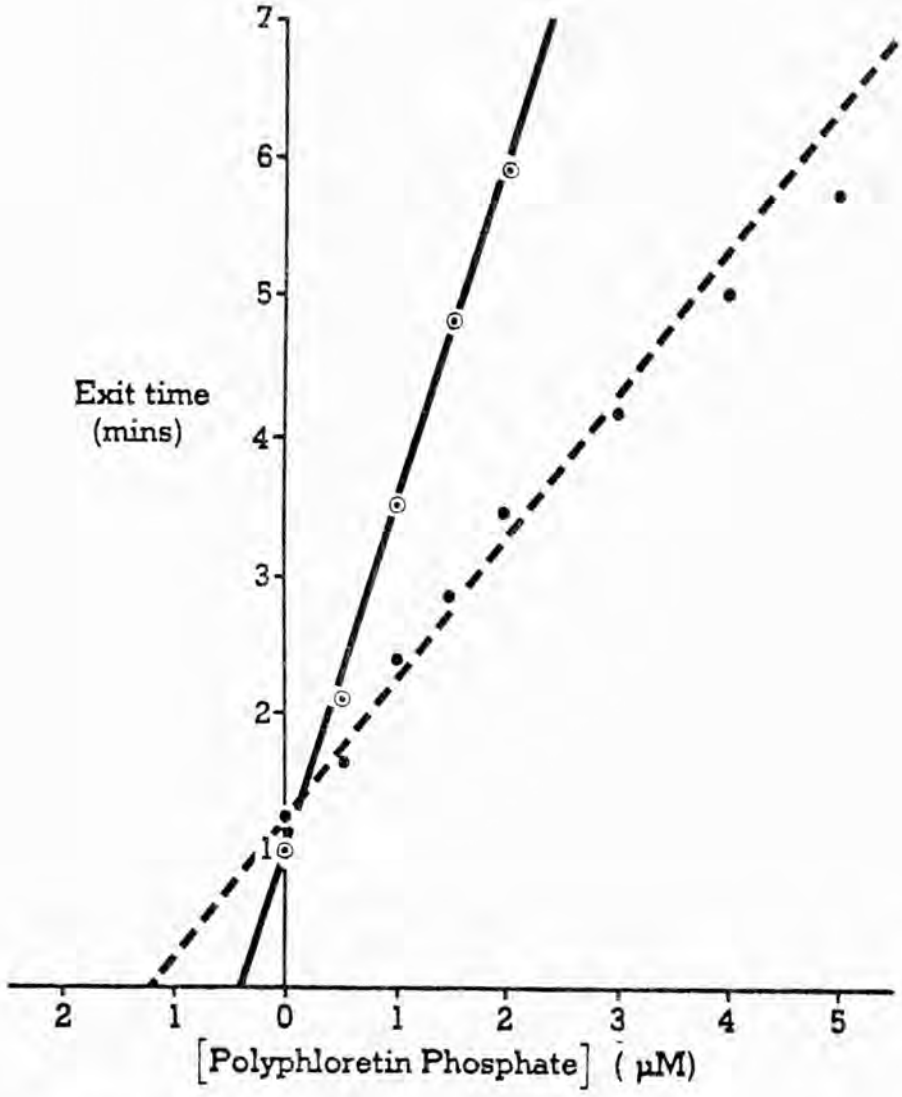


Figure 6.7

The effect of polyphloretin phosphate concentration on the glucose exit time from fetal and newborn guinea-pig erythrocytes at 16 °C. Exits were measured in a photoelectric apparatus with cells preincubated to contain 76 mM glucose. The interrupted line represents the data for human cells.

An estimate of the true K_I was made by extrapolating, to zero phlorizin concentration, the apparent K_I's estimated at each of the phlorizin concentrations, between 0.05 and 0.5 mM. The true K_I for newborn guinea-pig cells was estimated to be 0.053 mM and for human cells it was estimated to be 0.095 mM. The V_{MAX} value remained unaffected by the presence of the inhibitor in a typical competitive fashion.

3. Experiments with Polyphloretin Phosphate

(MW - average 4600)

Figure 6.6 is a typical plot of glucose exit inhibition by different concentrations of polyphoretin phosphate at 16 ^{o}C, for both newborn guinea-pig and human red blood cells. This plot shows a characteristic competitive response, whereby the intercept on the abscissa, which represents the half-saturation concentration, is seen to increase with corresponding increase in the inhibitior concentration.

The K_I, as measured by Sen-Widdas exit technique, for newborn guinea-pig cells was estimated to be ca. 0.45 μM. It was ca. 0.94 μM for human cells (see Figure 6.7).

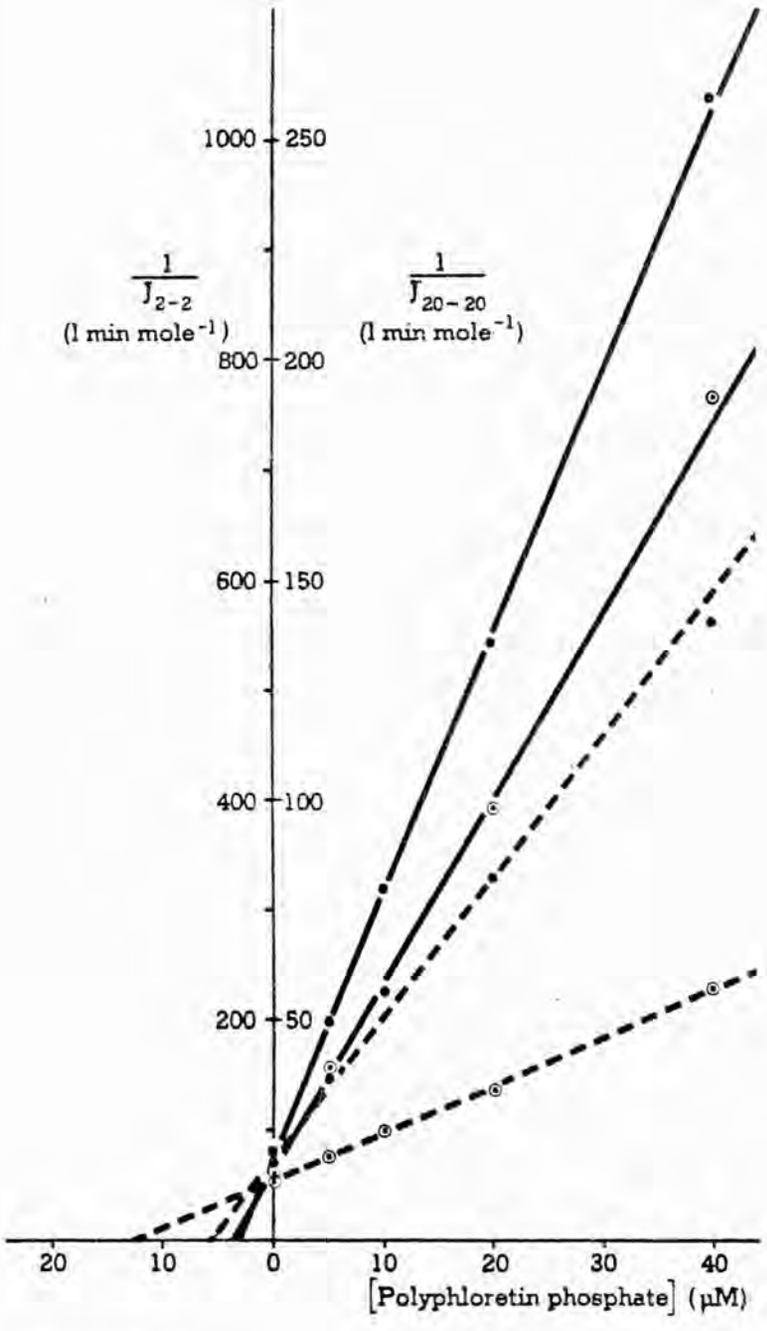


Figure 6.8

The effect of polyphloretin phosphate concentration on 3-O-methyl glucose exchange at 16 ^{o}C in fetal and newborn guinea-pig red cells (solid lines) and human cells (interrupted lines). Points ●, 2 mM exchange; points ⊙, 20 mM exchange. Points are a mean of two similar results at each sugar concentration.

Table VIii

Sen-Widdas exits and equilibrium exchange inhibitory constants for biphenolic compounds with newborn guinea-pig and human red blood cells, measured at 16 ^{o}C.

Inhibitor	Blood	Conc^{n} which half inhibits glucose exits (S-W constant) (μM)	Exchange inhibitory constant (μM)	Ratio: Exchange Constant / S-W Constant	Presumed site of action of inhibitor
Phloretin	Newborn guinea-pig	0.068	0.34	5:1	mostly outside
	Human*	0.24	0.4	1.67:1	mostly inside, some outside
Phlorizin	Newborn guinea-pig	50	53	1.1:1	mostly inside, some outside
	Human	80	95	1.2:1	mostly inside, some outside
Polyphloretin phosphate	Newborn guinea-pig	0.30	1.64	5.44:1	outside
	Human	0.94	5.8	6.2:1	outside

* Data from Basketter and Widdas (1978)

The 3-O-methyl glucose fluxes were inhibited by polyphloretin phosphate, in both newborn guinea-pig and human cells and the inhibition of 2 mM 3-O-methyl glucose by various inhibitor concentrations is shown in Figure 6.8. The inhibition of 2 mM and 20 mM 3-O-methyl glucose exchange by various concentrations of polyphloretin phosphate, together with the inhibition of 3-O-methyl glucose, at different concentrations, by 5 μM polyphloretin phosphate were all consistent with an estimated K_I of ca. 1.64 μM for newborn guinea-pig red cells and ca. 5.8 μM for human cells.

In conclusion, therefore, the three biphenols, phloretin, phlorizin and polyphloretin phosphate, have all been shown to inhibit the hexose transport systems from red blood cells in both newborn guinea-pigs and humans, in a characteristically competitive fashion. The kinetic parameters are summarised in Table VIii.

The ratio of the K_I values for the inhibition of exchange to that for the inhibition of Sen-Widdas exits was used by Basketter and Widdas (1978) as a pointer to the site of action of particular inhibitors. A high value of around 6 is typical of outside inhibition while a low value is characteristic of inside inhibition.

However, an intermediate value indicates that the inhibitor may be acting both inside and outside.

On the basis of reasoning by Basketter and Widdas (1978), it is noted that the exchange constant:S-W exits constant ratio is different for newborn guinea-pig and human cells, and that it is also different for different inhibitors.

A marked difference in this ratio value between newborn guinea-pig cells and human cells for phloretin is found. A value of 1.67 with human cells suggests that the inhibition occurs on both surfaces of the membrane, with the internal site providing the major contribution. A high ratio of 5:1 with newborn guinea-pig cells strongly suggests that most of the inhibition must occur on the outer surface of the cell membrane.

Striking similarity is shown by cells from both species with respect to the site of inhibition of the hexose transport system by phlorizin. Phlorizin inhibition is presumed to be exerted mainly from the inside of red cells in both the species, with some contribution from the external surface sites.

The low ratio for phlorizin may be seen as kinetic evidence for the ability of phlorizin to penetrate the red

cell membrane. Phlorizin has been, earlier, shown not to penetrate the cell membrane in hamster intestine (Stirling, 1961) and renal proximal tubule luminal membrane (Frasch, 1970; Glossmann and Neville Jr., 1972).

The similarity in terms of site of action of phlorizin inhibition in cells from both newborn guinea-pigs and humans and the difference in terms of site of action of phloretin, in these cells, would seem to rule out the possibility that the action of phlorizin is accountable by phloretin contamination of phlorizin and/or hydrolysis of phlorizin to phloretin, as suggested by LeFevre and Marshall (1959).

The finding that polyphloretin phosphate acts exclusively on the outer surface of the human red cell membrane is suggested by the very high ratio of 6.2:1. This is in agreement with the earlier finding of polyphloretin phosphate's inability to penetrate this membrane (Wilbrandt, 1954). The high ratio of about 6 is characteristic of maltose, which inhibits the hexose transfer system exclusively from the outside (Basketter and Widdas, 1978).

Similar conclusions can also be drawn from the findings with newborn guinea-pig cells and polyphloretin phosphate. The high ratio of 5.4:1 is again suggestive of an inhibitor acting solely on the outside surface of the cell membrane. Thus this finding is consistent with the view that polyphloretin phosphate is unable to penetrate the newborn guinea-pig erythrocyte as well.

CHAPTER 7

INHIBITION OF HEXOSE TRANSFER BY 1-HALO-2,4-DINITROBENZENES

Results

Inhibition of Hexose Transfer by 1-Halo-2,4-Dinitrobenzenes

The hexose transfer system in the human erythrocyte is non-competitively and irreversibly inhibited by 1-Fluro-2,4-Dinitrobenzene (FDNB) (Bowyer and Widdas, 1956; Krupka 1971). The inhibition by FDNB proceeds more rapidly in the presence of glucose. The acceleration of inhibition by sugars is found to be saturable and the concentration giving half the maximal effect corresponds reasonably with the concentration of glucose which half saturates the hexose transfer system at that temperature. This effect is contrary to what would be expected if both FDNB and the glucose were interacting at the same site on the cell membrane (Bowyer and Widdas, 1958). Bowyer and Widdas (1958) also put forward the suggestion that this effect was a consequence of the reaction of glucose with the membrane component , which leads to the exposure of FDNB reactive site, thus facilitating the FDNB reaction.

The phenomena of acceleration of the FDNB reaction with sugars which react with the hexose transfer system, is not confined to glucose, but is also exerted by other sugars in a specific manner. The most readily transported sugars accelerate inactivation most, for instance 2-deoxyglucose accelerates inactivation up to five fold whereas maltose, which can combine with the carrier but is not transported actually protects the system from inactivation. It is considered that a transportable sugar has the ability

to stabilize the carrier in one conformation whilst maltose and other such protective compounds are thought to stabilize the carrier in another conformation. Competitive inhibitors, phloretin and phlorizin have opposite effects. The former reduces the FDNB reaction rate while the phlorizin increases it (Krupka, 1971a).

That the site with which FDNB reacts plays an essential role in the re-orientation of the carrier and that this site may not be involved in sugar binding was suggested by Krupka and Déves (1980). Also the FDNB reactive site cannot be located at the same site as the sugar since it reacts more rapidly when a transportable substrate is bound to the carrier.

Krupka and Déves (1980) have taken the study of FDNB interaction with the hexose carrier one step further in that they undertook an investigation of the reactions of the hexose carrier with a series of 1-Halo-2,4-Dinitrobenzenes. They found that the fluoro, choro, bromo and the iodo analogs all acted as non-competitive, irreversible inhibitors and that all these compounds reacted at the same site, since Cytochalasin B protects against inactivation by these compounds and that these halo compounds do not have any general effects on the structure of the membrane, or permeation systems thereof, eg choline or glycerol.

There is however, a very striking difference between

the FDNB and the other three nitrobenzenes, with respect to their inhibition reaction rates in the presence and absence of sugars. Although FDNB inhibition is facilitated by the presence of a transportable substrate, the chloro, bromo and the iodo analog reactions are not. It is found that FDNB is the least reactive of the four and that IDNB is the most reactive towards the hexose carrier. This is just the opposite to what would be expected of these compounds from their reactions with proteins and most other chemical reactions, where the fluoro compound is usually found to be the most reactive. This reverse order must therefore result from special properties of the reaction site in the carrier (Krupka and Devés, 1980).

Comparison of the general base catalysis of N-methyl-aniline with these halo-compounds and the occurrence of substrate stimulation with FDNB but not with the chloro, bromo or the iodo nitrobenzenes suggests that binding may promote a general base catalysis and as the reactive group in the carrier is thought to be an amine, the possibility of a general base catalysis playing a part in transport was put forward (Krupka and Devés, 1980).

In conclusion, Krupka and Devés (1980) put forward the hypothesis that the halo-dinitrobenzene site is probably involved in the carrier re-orientation rather than substrate binding.

It was thus thought of interest that this present study of comparison of the hexose transfer systems from the erythrocytes of humans and fetal and newborn guinea-pigs

should involve a comparison of the 1-halo-2,4-dinitrobenzene reaction with this system. Of the possible halodinitrobenzenes, for which the inhibitory reaction does not seem to be facilitated by the presence of sugar, BrDNB has been chosen as a representative of this group.

In the present work, attempts were made to estimate the inhibition of the hexose transport in red blood cells, from the two species, produced by FDNB and BrDNB. The amount of inhibition produced was measured using the Sen-Widdas exits procedure and also the equilibrium exchange procedure.

The effect of the presence and the absence of 76 mM 2-deoxyglucose on the inactivation rate of these inhibitors was likewise measured by the above techniques.

In all experiments, the red blood cells were treated in an identical manner. The reaction of either FDNB or BrDNB was carried out at a concentration of 1.4 mM and at 22 ^{o}C for 15 minutes. The glucose exits were performed at either 16 ^{o}C or 27 ^{o}C and all equilibrium exchange measurements at 16 ^{o}C.

For the comparison of the inactivation rates, the method of Krupka (1971a, b) was followed. The inactivation rate constant k' was calculated from the the relationship:

$$\frac{T}{T_o} = e^{k'[I]t} \quad \text{or} \quad 2.303 \log\frac{T}{T_o} = k'[I]t \qquad (7.1)$$

where T and T_o are the exit times for the inhibited cells

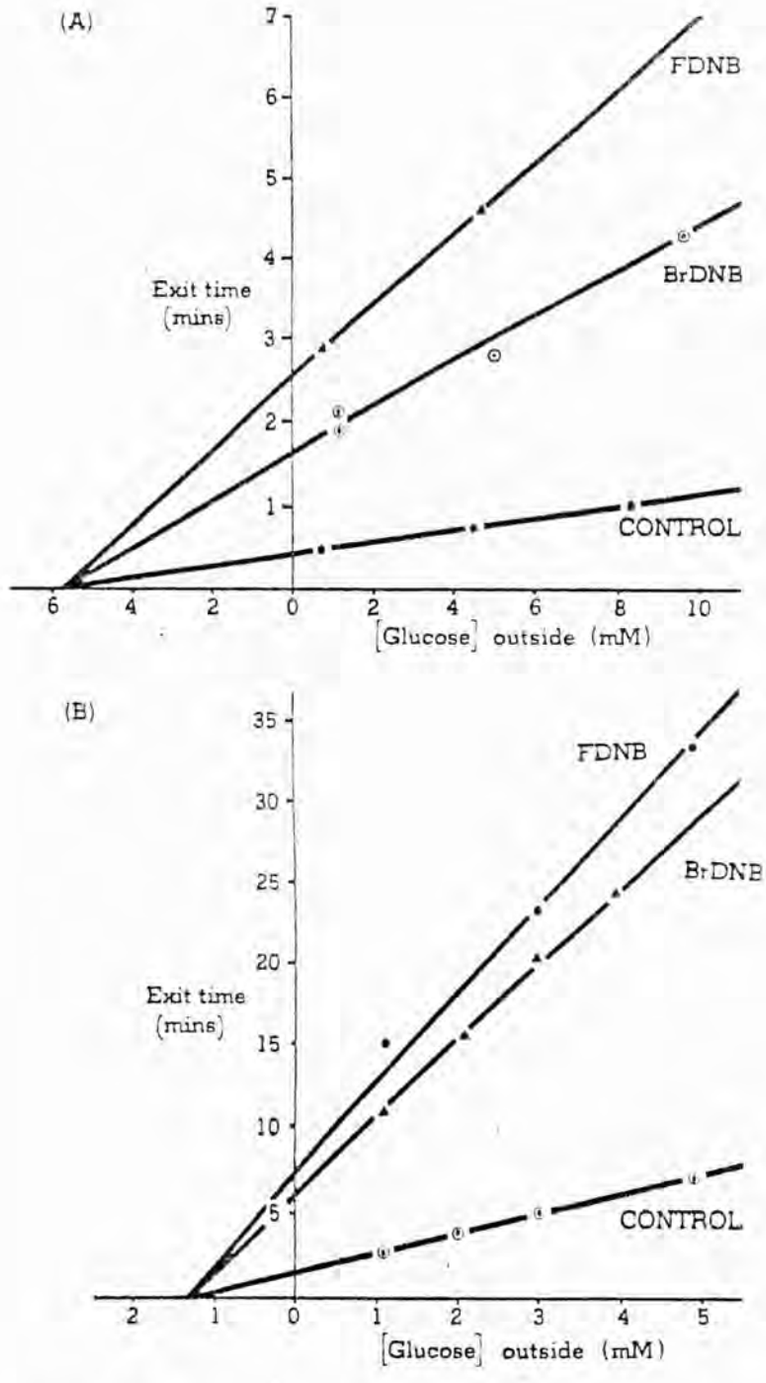


Figure 7.1

Inhibition of the glucose exits by FDNB and BrDNB in fetal and newborn guinea-pig (A) and human (B) erythrocytes. The cells were incubated with 1.4 mM FDNB and BrDNB, respectively for 15 minutes at 22 ^{o}C, pH 7.4 in the presence of 76 mM 2-deoxyglucose. Glucose exits from fetal and newborn guinea-pig cells were measured at 27 ^{o}C while those from human cells were measured at 16 ^{o}C. The results are typical of experiments with these inhibitors.

and the uninhibited cells respectively. I is the concentration of the inhibitor and t the time taken in minutes of incubation. For exchanges the reciprocal of the fluxes were used in place of exit times in Equation 7.1.

Results

The similarity of the inhibitory action of FDNB and BrDNB can be readily observed by plotting the exits times against the outside glucose concentrations, for cells inhibited by these inhibitors and also for uninhibited cells as controls.

The action of both FDNB and BrDNB with newborn guinea-pig and human cells is shown characteristically to be of the non-competitive type, with glucose exits, Figures 7.1A and B. The half-saturation concentration for glucose remains unaltered in the presence of the two inhibitors, however the exit time for glucose into glucose-free medium in increased. This increase is, for example, by a factor of about 4.8 and 4.0 by FDNB and BrDNB respectively in human cells, at 16 ^{o}C.

The hexose transfer system across the human erythroctye membrane is inhibited to a similar degree by both the FDNB and the BrDNB. There is a 66.9% inhibition of this system by 1.4 mM FDNB, incubated for 15 minutes at 22 ^{o}C, pH 7.4 in the presence of 76 mM 2-deoxyglucose and a 59% inhibition by 1.4 mM BrDNB under matched conditions, for Sen-Widdas extis at 16 ^{o}C.

A similar behaviour is observed with fetal and newborn guinea-pig red blood cells. The glucose exits

Table VIIi

The effect of the presence and absence of 76 mM 2-deoxyglucose on the inactivation rate constant and % inhibition by 1.4 mM FDNB and 1.4 mM BrDNB, incubated for 15 minutes, at 22 ^{o}C, pH 7.4, as measured by the Sen-Widdas exits procedure.

Blood	Inhibitor	2-deoxy-glucose	k' (ℓ mole^{-1} min^{-1})	% Inhibition
Human	FDNB	+	54	66.9
	FDNB*	-	9	16.7
	BrDNB	+	43	59.0
	BrDNB	-	38	55.0
Newborn guinea-pig	FDNB	+	92	85.5
	FDNB	-	68	75.4
	BrDNB	+	77	79.7
	BrDNB	-	119	91.7

The k' value for human and newborn guinea-pig cells was calculated from glucose exit measuremnts made at 16 ^{o}C and at 27 ^{o}C respectively.

* Results of a single experiment

in these cells were 85.5% inhibited with FDNB and 79.7% with BrDNB. The maximal transfer rate, in this case, is reduced by a factor of about 5.7 with FDNB. The corresponding maximal transfer rate with BrDNB is reduced by a factor of only about 3.7.

Further experiments, to compare the actions of FDNB and BrDNB with respect to the facilitation of inactivation by the presence of a sugar, were carried out. In this situation the red blood cells were incubated with either 1.4 mM FDNB or 1.4 mM BrDNB at 22 ^{o}C for 15 minutes in the presence and in the absence of 76 mM 2-deoxyglucose.

Following the initial incubation of red cells with appropriate inhibitor, the cells were washed of both excess inhibitor and, where applicable, the 2-deoxyglucose by first separating the cells from the incubating medium by centrifugation and then resuspending the cells in 10 ml phosphate buffered saline, followed by a further 5 minute incubation, at room temperature. The cells were separated and the procedure repeated before glucose exits or equilibrium exchange measurements were carried out.

The results of Sen-Widdas exit experiments are summarised in Table VIIi.

Throughout this study, the glucose exits and equilibrium exchange measurements were arranged such that comparisons of the effect of presence and absence of sugar on inhibition by FDNB and BrDNB, respectively, were carried out on

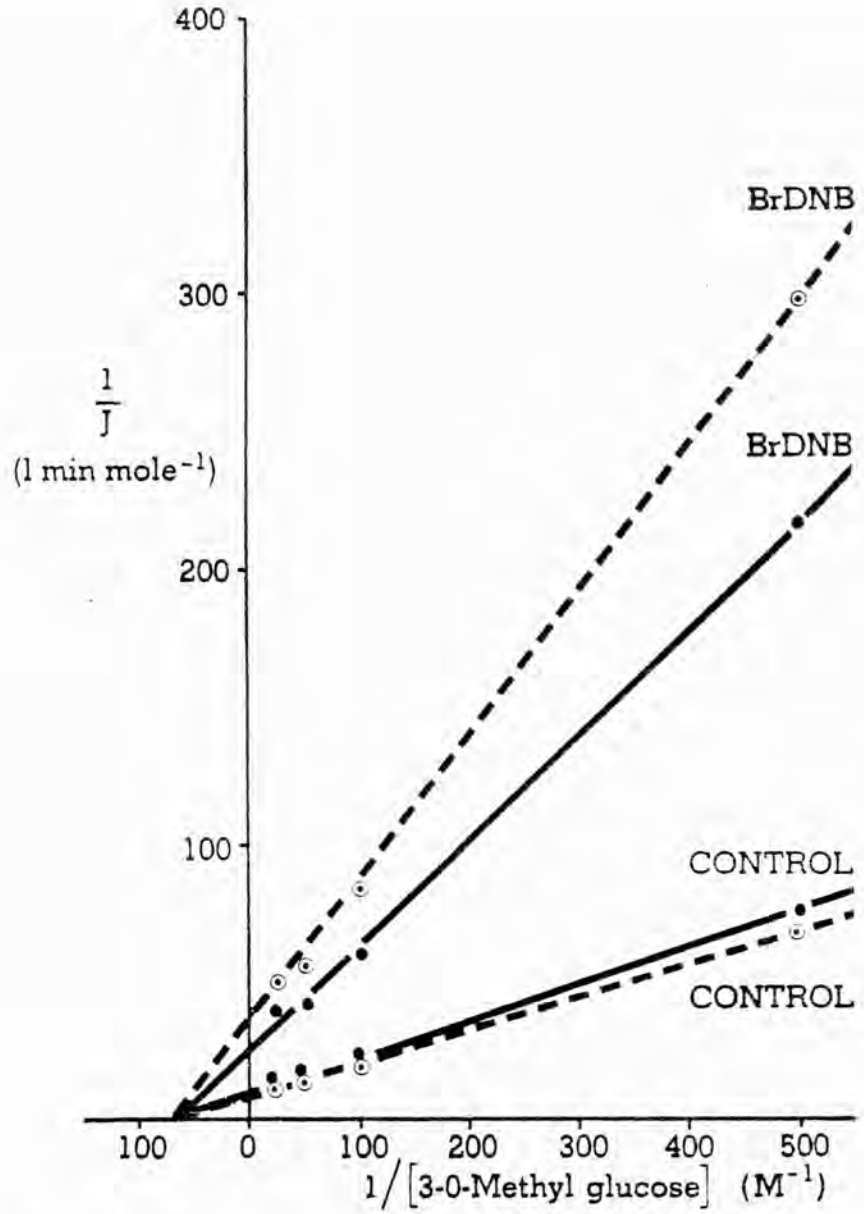


Figure 7.2

The inhibition of 3-O-methyl glucose exchange fluxes in fetal and newborn guinea-pig erythrocytes at 16 °C by 1.4 mM BrDNB incubated for 15 minutes at 22 °C, pH 7.4, in the presence of 76 mM 2-deoxyglucose. The interrupted lines represent the corresponding results for human cells. Points are a mean of two experiments with similar results.

Table VIIii

The effect of the presence or absence of 76 mM 2-deoxyglucose during incubation with halo-dinitrobenzene on % inhibition of 20 mM 3-O-methyl glucose equilibrium exchange fluxes. Cells were incubated with 1.4 mM FDNB and BrDNB, respectively for 15 minutes, at 22 ^{o}C, pH 7.4.

Blood	Inhibitor	2-deoxy-glucose	k' (ℓ mole^{-1} min^{-1})	% Inhibition
Human	FDNB	+	83	83.2
	FDNB	-	22	31.1
	BrDNB	+	67	76.9
	BrDNB	-	60	71.8
Newborn guinea-pig	FDNB	+	68	77.4
	FDNB	-	45	62.3
	BrDNB	+	47	67.9
	BrDNB	-	74	79.4

the same sample of blood on the same day, thus minimizing factors which may otherwise introduce errors. Similarly, the comparison of inhibition by these inhibitors on human or newborn guinea-pig cells were carried out on the same day.

The results of inhibition of exchange by BrDNB in cells from both species is shown in Figure 7.2.

The equilibrium exchange of 3-O-methyl glucose is also noncompetitively inhibited by both FDNB and BrDNB in the human and newborn guinea-pig cells. Equilibrium exchange fluxes are inhibited to a similar degree by both inhibitors in the humans however, for newborn guinea-pig cells the BrDNB results are somewhat anomalous. The results are summarised in Table VIIii

Inactivation of equilibrium exchanges by FDNB is affected by the presence of 2-deoxyglucose, in the incubation medium, more so in human erythrocytes than in newborn guinea-pig red cells. There is almost 2½ times as much inhibition of the 3-O-methyl glucose exchange by 1.4 mM FDNB in the presence of 76 mM deoxyglucose, incubated for 15 minutes at 22 ^{o}C than there is in its absence. In the corresponding inhibition of newborn guinea-pig cells, there is only about 25% increase in the presence of the sugar.

The presence of sugar during BrDNB incubation does not seem to have any affect on the amount of inactivation in human cells. In the newborn guinea-pig cells, BrDNB inhibits the cells slightly more in the absence of sugar than in its presence.

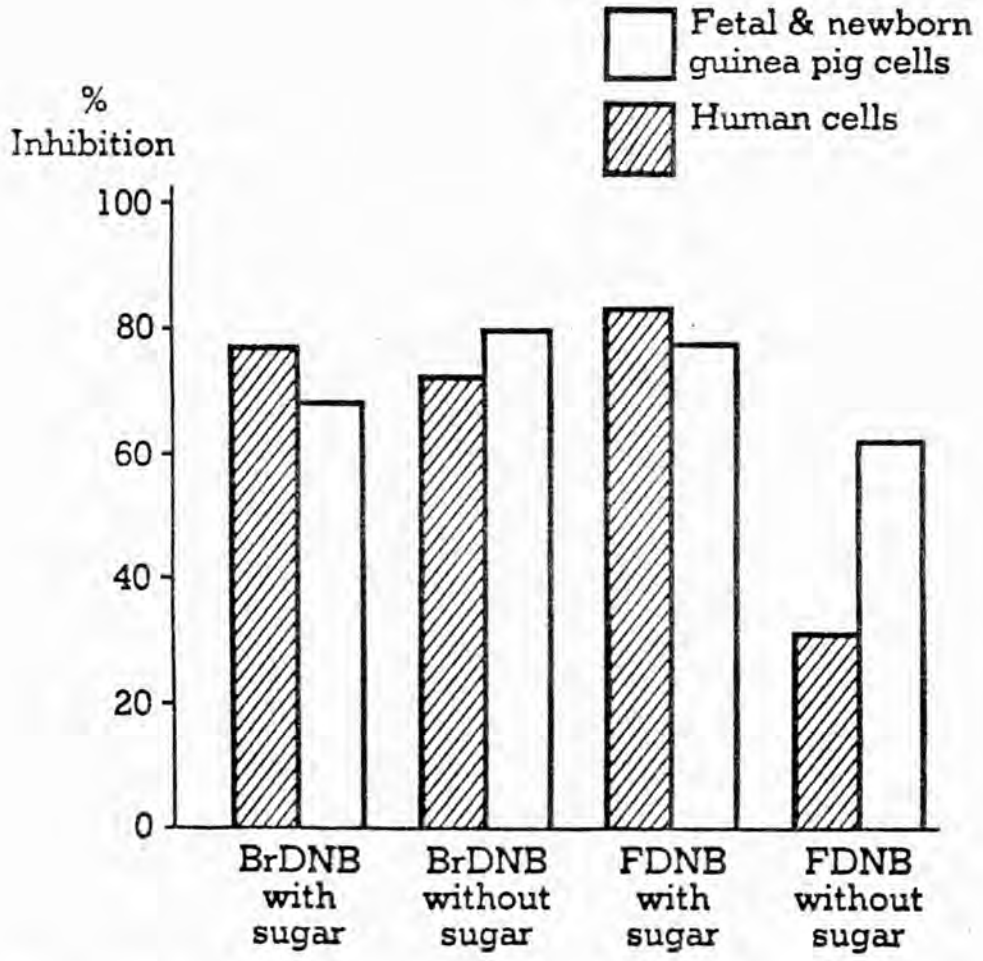


Figure 7.3

The effect of the presence and the absence of 76 mM 2-deoxy-glucose on percent inhibition of 20 mM 3-O-methyl glucose exchange fluxes, by 1.4 mM FDNB and 1.4 mM BrDNB respectively incubated for 15 minutes at 22 ^{o}C, pH 7.4, with erythrocytes from fetal and newborn guinea-pigs and adult humans.

Thus whereas 2-deoxyglucose enhances the inactivation rate of the hexose transfer system by FDNB in both human and newborn guinea-pigs there is no analogous facilitation of inactivation with BrDNB.

It is tempting to suggest that the presence of sugar during BrDNB reaction with the cells has the effect of protecting the hexose transfer system against inactivation, in as much as the equilibrium exchange of 3-O-methyl glucose in the newborn guinea-pig cells is inhibited 11.5% less in the presence than in the absence of sugar. This feature is also suggested qualitatively, by results obtained from measuring glucose exits.

The acceleration of inactivation by FDNB in the presence of 2 deoxy-glucose, which is so conspicuous with human cells is however, not very apparent with newborn guinea-pig cells, see Figure 7.3. The inactivation rate by FDNB with human cells is enhanced by a factor of about 2.7 whereas it is only enhanced by a factor of 1.2 with newborn guinea-pig cells.

The most prominent feature observed during experiments with these non-competitive inhibitors was that the inhibition of newborn guinea-pig cells by FDNB was not accelerated to a similar degree by the presence of 2-deoxyglucose as was the transport system in human cells. This observation suggests that the hexose transport system in newborn guinea-pigs may be such that the FDNB reactive site may not require the degree of exposure, associated with the conformational change during translocation, that is required by human cells.

It may therefore follow that the FDNB/BrDNB binding site must be extremely close to the sugar binding site, thus the second observation, that of protection by sugar against the inhibitory reaction of BrDNB in guinea-pig cells, would be consistant with the hypothesis that the sugar bound to the transport site interferes with the more bulky BrDNB molecule, by steric hinderence, but not with the smaller FDNB molecule.

CHAPTER 8

DISCUSSION

1. Introduction
2. Kinetic parameters of hexose transfer
3. Effect of non-transportable inhibitors on hexose exchange
4. Inhibition of hexose exchange with Cytochalasin B
5. Cytochalasin B binding to erythrocyte membranes
6. Inhibition of hexose transfer by various biphenols
7. Inhibition of hexose transfer with Halo-2,4-dinitrobenzenes
8. Possible physiological function of the hexose transfer system in fetal erythrocytes
9. Possible future investigations

Discussion

The movement of glucose across cell membranes is only a small function of the membranes wider variety of activities associated with its structure. Membranes are involved in functions such as the cell-cell interaction, contractile processes, excitation in nerves, ATP generation and so on. Many of these processes are in turn closely related to the movements of ions and other substances across the membrane.

The hexose transfer system has been conveniently studied in human red blood cells, however comparative studies in other species and tissues have indicated that this system has a wider biological significance.

Taking various factors into account, such as the post-natal changes in membrane proteins in relation to the hexose transfer system (Jung et al., 1980), changes in the hexose transfer after birth (Widdas, 1955), etc., it is assumed that the newborn guinea-pigs of the age used in this study had few neonatal red cells present in the circulation. It must be remembered that the red blood cells of both humans and in particular the fetal and newborn guinea-pigs do not constitute a homogeneous cell population. It is however, remarkable that the results of experiments with these cells are highly repeatable.

The purpose of the experiments reported here was to make a further contribution to the understanding of the hexose transport across red cell membranes, in the first

place by comparing the hexose transfer system in fetal and newborn guinea-pig red cells with the similar system in adult human red cells, and in the second instance, by providing raw kinetic data for the hexose transport in fetal and newborn guinea-pig erythrocytes.

The kinetic data has been obtained by well established procedures, thus it is hoped that this would tend to minimise disagreement in the interpretation of data, so obtained.

Kinetic Parameters of Hexose Transfer

The kinetic parameters for the hexose transfer system, in both fetal and newborn guinea-pig and human cells, have been measured using two experimental procedures. These two procedures were the Sen and Widdas exits technique and the equilibrium exchange procedure.

Two different sugars, D-glucose and 3-O-methyl-D-glucose, have been used to measure the kinetic parameters with the two different experimental procedures. Glucose has been used for the Sen-Widdas exits procedure while 3-O-methyl glucose has been used in the equilibrium exchange experiments. The primary reason for using glucose is simply that it is the sugar that has been used by almost every worker in this field of study thus comparisons of results are possible. Secondly, the Sen-Widdas exits procedure entails measuremnts of efflux of sugar from cells preloaded to a very high glucose concentration. Thus any change in this concentration due

to metabolism that may occur during the experimental run would introduce insignificant errors, since the half-saturation concentration (≈1.5 mM for glucose) is very low compared with the concentration to which the cells are preloaded (≈76 mM). In the case where equilibrium exchanges have to be measured at very low sugar concentration (≈2 mM), the reduction in sugar concentration, by metabolism, would introduce significant errors. Thus the widely used non-metabolisable glucose analogue, 3-O-methyl glucose, with a relatively high affinity for the hexose transfer system, was chosen to characterise the membrane transport system with regards to the sugar-sugar exchanges between the inside and the outside of the cells. The metabolically inert nature of 3-O-methyl glucose was shown by Crane (1960) while studying the intestinal absorption of sugars.

The Sen and Widdas exit procedure has been visualised as giving the half-saturation concentration for the outside sites. Similarly, on the basis of kinetics discussed earlier, the equilibrium exchange, in the absence of any inhibitor is depicted as giving a half-saturation constant which approaches a value equal to the higher valued site. For human cells, the inward facing hexose transfer site has the higher half-saturation concentration for most common monosaccharides.

Thus on the basis of the above assumptions, the glucose half-saturation concentration for the outside sites, for fetal and newborn guinea-pig cells was ca. 2.7 mM

and the maximal transfer rate was ca. 0.26 isotonic units min^{-1}, at 16 ^{o}C and pH 7.4. The corresponding values for the human erythrocytes were ca. 1.5 mM for the half-saturation concentration and ca. 0.15 isotonic units min^{-1} for the maximal transfer rate. As far as the outside site is concerned in the transfer system in fetal and newborn guinea-pig cells, the carrier-sugar interaction would seem to be less tenacious than the similar interaction of glucose with the membrane component in human cells. The maximal transfer rate, which is higher for fetal and newborn guinea-pig erythrocytes suggests that either the hexose transfer system in these cells is present in a greater quantity than in human cells or that the transfer component is able to work much faster in fetal and newborn guinea-pigs than in humans.

The comparison of above parameters was earlier made by Dawson and Widdas (1964) with respect to their variations with temperature and pH. They measured the kinetic parameters according to Sen and Widdas (1962) and found that the pH dependence of the hexose transfer system was similar in both fetal guinea-pig and human erythrocytes. They also showed that the half-saturation constant, similarly obtained, decreased with decrease in temperature, but became relatively temperature independent below about 30 ^{o}C. The fall in the half-saturation constant, with temperature, was not the same in cells from the two species. The maximal transfer rate was found to be greater in fetal guinea-pig blood cells

than in human erythrocytes and the variations of this, with temperature, was essentially similar in the two species.

The equilibrium exchanges of sugars across the fetal and newborn guinea-pig erythrocyte membrane has not been investigated previously. In the present work it was found that the equilibrium exchange of 3-O-methyl glucose was faster in newborn guinea-pig cells than in the human. The half-saturation concentration for 3-O-methyl glucose exchanges was estimated at 16 ^{o}C and was found to be ca 25 mM for the fetal and newborn guinea-pig cells, which compares with ca. 15 mM for human cells. The maximal transfer rate at this temperature was ca. 167 mmole ℓ^{-1} min^{-1} for the fetal and newborn guinea-pigs and ca. 119 mmole ℓ^{-1} min^{-1} for human cells.

The half-saturation concentration and the maximal transfer rate, determined from the exchange experiments, was higher in fetal and newborn guinea-pig cells than human cells so that at 20 mM 3-O-methyl glucose the equilibrium exchange rates were similar in the two species.

Thus the studies with sugar-sugar exchange show a comparable variation with those from the Sen-Widdas experiments, that is to say, a faster rate and a lower affinity, in the hexose transfer system in erythrocytes from fetal and newborn guinea-pigs.

Effect of Non-transportable Inhibitors on Hexose Exchange

Non-transportable inhibitors of the hexose transfer system allow a direct comparison between inhibition exerted solely from the outside or exclusively from the inside of the red cells. This is possible since this group of inhibitors has been found to inhibit the transfer system without itself utilizing it for penetration, at the same time these inhibitors are able to penetrate the red cell membrane by simple diffusion, at a very low rate.

Thus with ethylidene glucose, present exclusively on the outside of the cells, the inhibition of 20 mM 3-O-methyl glucose exchange flux at 16 ^{o}C was in accordance with a K_I of ca. 10 mM with newborn guinea-pig cells. The exchange K_I, with ethylidene glucose present solely on the inside, was ca. 52 mM. Thus, this difference in affinities for the hexose transfer system at the two sides of the membrane indicates an apparent fivefold asymmetry for ethylidene glucose, in the transport system. A greater, but similar asymmetry has earlier been reported with human erythrocytes, where the K_I for ethylidene glucose on the inside was ca. 110 mM and was ca. 11 mM for the outside site (Baker et al., 1978).

The above is therefore evidence for an asymmetry of affinities in the hexose transfer system in fetal and newborn guinea-pig erythrocyte membrane, comparable with a larger tenfold asymmetry of affinity encountered in adult human erythrocytes.

Trimethyl glucoside further supports the asymmetry of affinities by having a higher affinity for the inward facing site compared with the affinity for the outside transfer site. The exchange K_I for trimethyl glucoside, at 16 ^{o}C, for the inward facing site was ca. 140 mM and for the outside site it was ca. 170 mM, for fetal and newborn guinea-pig erythrocytes. The corresponding values for human erythrocytes, were ca. 113 mM for the insdie sites and ca. 290 mM for the outside sites.

The observations with red blood cells from fetal and newborn guinea-pigs and adult humans therefore show that the hexose transfer system present in these cells are qualitatively similar in terms of asymmetry of affinities.

The existence of differences in affinities on the two sides of the membrane suggests that the opposing 'carrier' sites are distinct from one another, thus this finding is in contradiction to some models for hexose transfer which propose a symmetrical system.

The kinetics to describe asymmetry in hexose transfer systems from erythrocytes were developed by Regen and Morgan (1964), Geck (1971), Regen and Tarpley (1974) and Baker and Widdas (1973b). In order that the model should take into account asymmetry of affinities and uneven mobilities of the unsaturated and the saturated carriers, without leading to accumulation of sugar on one side of the cell membrane, the necessary provision for redistribution of carriers has to be made (Widdas, 1952).

Thus a consideration of redistribution of carriers, between the two sides of the cell membrane, becomes compulsory when dealing with interpretation of results of experimental situations where there may be a non-equilibrium sugar distribution across the cell membrane or in a situation where an inhibitor may be acting on, or predominantly on one side.

In the experiments of the present work, the conditions were nearly all designed to have sugars at equilibrium at the two sides and inhibitors in reasonably low concentrations so that redistribution effects were not apparent. Further the main purpose was to compare results obtained with fetal and newborn guinea-pig cells with those obtained using human red cells in experiments carried out at similar concentrations so that any second order effects due to redistribution would apply to both systems.

Cytochalasin B

1. Inhibition of Hexose Transfer

There is no great, or very significant, difference observed between the inhibition of the hexose transfer system in fetal and newborn guinea-pig and adult human erythrocyte by Cytochalasin B.

The hexose transfer system, in fetal and newborn guinea-pig red blood cells is inhibited by Cytochalasin B, at 16 ^{o}C, with a K_I of ca. 0.42 μM when measured by the Sen-Widdas exits technique and a K_I of ca. 0.24 μM when

measured by the inhibition of equilibrium exchange fluxes.

The inhibition of the hexose transfer system, in adult human erythrocytes, by Cytochalasin B, at 16 ^{o}C, proceeds with a K_I of ca. 0.5 μM and ca. 0.11 μM when measured by the Sen-Widdas and equilibrium exchange techniques, respectively.

Thus the inhibition of glucose exits in fetal and newborn guinea-pig erythrocytes is greater than that in human cells but the inhibition of exchange is slightly less in the former. The inhibition of hexose exchange is more effective than the inhibition of exits. This may be what would be expected if the inhibitor were acting inside the cells, since the inhibitor in the exits procedure would have to compete with a very high concentration of sugar inside the cells, hence the estimated K_I by this procedure would of course be larger than the K_I estimated by the inhibition of equilibrium exchanges. Similarly, for the inhibitor acting solely on the outside surface of the cell membrane, it would be expected for the K_I measured by the Sen-Widdas exits procedure to be very much smaller than that measured by the equilibrium exchange procedure. The latter has been confirmed with Maltose (Basketter and Widdas, 1978) which is restricted to the outside medium.

The nature of inhibition of the Sen-Widdas exits by Cytochalasin B would in the first instance indicate that the inhibition was of the non-competitive type whilst the inhibition of equilibrium exchange would indicate it to be of the competitive type. However, earlier it had been

pointed out that the Sen-Widdas exits technique only shows a change in the half-saturation constant in the presence of a competitive inhibitor which acts on the outside site while inhibition on either side of the cell membrane would be clearly shown by the equilibrium exchange procedure. Thus, this apparent non-competitive inhibition of glucose exits and the apparent competitive inhibition of equilibrium exchanges, together with the fact that the inhibition of exchange is more effective than the inhibition of exits, can be seen as evidence for a reaction between Cytochalasin B and the inward facing hexose transfer site only. Thus only the exchange procedure can be used to distinguish competitive inhibition from non-competitive inhibition.

This marked preference of Cytochalasin B to interact only at the inside site of the hexose transfer system in erythrocytes must, therefore, be strong evidence that this system is not identical at the two surfaces of the cell membrane. The difference in reactiveness of Cytochalasin B on the inside and the outside has also been shown by Devés and Krupka (1978). In chick embryo fibroblasts, Novikoff hepatoma cells and Hela cells, Cytochalasin B reacts with the outside sites only, thus Cytochalasin B may bind exclusively to either the inner or the outer transfer site, depending on the type of cell.

2. Cytochalasin B Binding to Erythrocyte Membranes

The binding of Cytochalasin B to fetal and newborn guinea-pig erythrocytes shows that it is taken up by two processes.

The total uptake of Cytochalasin B can be separated into a saturable and a non-saturable component. The reasons for assuming that the saturable Cytochalasin B binding correlates with the inhibition of hexose transfer have already been discussed, and on the basis of that hypothesis, the number of hexose transfer sites present in the fetal and newborn guinea-pig erythrocytes, have been calculated.

Thus the number of hexose transfer sites in erythrocytes from fetal and newborn guinea-pigs was 1.25×10^5 per cell. This estimation is almost equal to the lowest estimation of hexose transfer sites per human red blood cell (Masiak and LeFevre, 1972). Since the maximal transfer rate is ca. 167 mmole ℓ^{-1} min^{-1} and that there are 1.25×10^5 sites per cell, the turnover number for the hexose transfer "carriers" would therefore be 1.44×10^3 per second, at 16 ^{o}C.

The corresponding number of hexose transfer sites, also estimated using Cytochalasin B in human cells is 2.4×10^5 per cell and the maximal transfer rate is ca. 119 mmole ℓ^{-1} min^{-1}, thus the turnover number for hexose transfer in human erythrocytes at 16 ^{o}C is 0.5×10^3 per second (Data for human cells was collected from Basketter and Widdas, 1978).

The hexose transfer 'carrier' molecule is presumed to expose sites alternately between the two surfaces of the cell membrane thus the turnover number would presumably depend on the size of the carrier molecule and also the

viscosity of the membrane. However, since the hexose transfer system in fetal and newborn guinea-pig erythrocytes is very similar to that found in adult human erythrocytes (Aubby and Widdas, 1980), it is possible that the difference is chiefly in the microenvironment in which the "carrier" operates and it may be that the adult human red cell membrane is more viscous than that found in fetal and newborn guinea-pig erythrocytes.

To summarise, the Cytochalasin B inhibitory and binding studies have therefore revealed the following:

1. That the hexose transfer system in fetal and newborn guinea-pig erythrocytes is asymmetric and in that respect similar to the system present in human erythrocytes.
2. That there are some 1.25×10^3 hexose transfer sites per fetal and newborn guinea-pig erythrocyte.
3. That the turnover number of the hexose transfer site in fetal and newborn guinea-pig erythrocytes was 1.44×10^3 per second, compared with 0.5×10^3 per second in human cells.

The Cytochalasin B studies and the equilibrium exchange measurements were both made on newborn guinea-pig blood and are therefore comparable. It is interesting to note that Jung et al. (1980) found that cells possessing the fast hexose transfer system, similar to that present in human erythrocytes, remain essentially undiluted by adult-type cells for about eight days after birth in the rabbit.

It is interesting to note that the number of hexose transfer sites in human erythrocyte membranes have been estimated using p-chloromercuribenzene sulfonate (Van Steveninck et al., 1965), glucose (Stein, 1968), Cytochalasin B (Taverna and Langdon, 1973; Lin and Spudich, 1974; Jung and Rampal, 1976; Basketter and Widdas, 1977) and impermeant maleimide (Batt et al., 1976). A comparison of the number of transfer sites estimated by various authors is made by Basketter and Widdas (1978).

In concluding it is appreciated that to get a better quantitative comparison of the Cytochalasin B binding with the hexose transfer system in cells from the fetal and newborn guinea-pigs and adult humans, it may be necessary to eliminate any systematic errors introduced by using different batches of Cytochalasin B. Thus a future project would include a re-estimation of the Cytochalasin B binding with the use of cells from the two species in an experimental protocol which was otherwise identical.

Inhibition of Hexose Transfer by Various Biphenols

The behaviour of the biphenolic compounds phloretin, phlorizin and polyphloretin phosphate was studied to compare inhibition of hexose transfer in erythrocytes from fetal and newborn guinea-pigs and adult humans.

The fetal and newborn guinea-pig erythrocytes have a greater affinity for the three biphenols studied at

16 ^{o}C and pH 7.4 than human erythrocytes, both when measured by the Sen-Widdas exit method and also the equilibrium exchange procedure.

These inhibitors are all effective at very low concentrations except phlorizin, which inhibits the hexose transfer at concentrations almost two orders of magnitude higher than either phloretin or polyphloretin phosphate.

The most pronounced difference between the inhibition of hexose transfer in the two species was shown by phloretin. The Sen-Widdas exits were inhibited very effectively with a K_I of 0.068 μM in fetal and newborn guinea-pig cells compared with a K_I of 0.24 μM for human cells. The inhibition of exchanges by phloretin in the two species, on the other hand, shows relative similarity. The most obvious conclusion to be drawn from these results would be that phloretin acts mainly on the outer surface of the erythrocyte for fetal and newborn guinea-pigs while it acts partly on the internal surface of the human cell membrane.

The inhibition of the hexose transfer system in erythrocytes from the two species with phlorizin shows great similarity both in terms of exits and also exchanges. The similarity suggests that the site of action of phlorizin in these cells must be similarly located. In other words, the findings are suggestive of inhibition being exerted mainly from the inside of the cells with some contribution from externally inhibited sites. Thus

implicit in these findings is the suggestion that phlorizin is able to penetrate the red cell membrane in both fetal and newborn guinea-pigs and humans. This finding is in direct contrast to the report that phlorizin inhibits the carrier-mediated ion exchange across human erythroctyes only from the outside, but not from the inside (Lepke and Passow, 1973; Schnell et al., 1973).

The observation that the apparent half-saturation constant for phlorizin increased with increasing inhibitor concentration, as described earlier in Chapter 6, may also be seen as a possible clue to the proposal that phlorizin exerts most of its inhibition from the inside of the erythrocytes, since a finite time would be required for a majority of the phlorizin to penetrate the red cell membrane to exert its inhibition fully.

The inhibitory studies with polyphloretin phosphate support the earlier findings of Wilbrandt (1954) that polyphloretin phosphate was unable to penetrate the red cell membrane. This is indicated by the finding that the inhibition of the Sen-Widdas exits was much more effective than exchanges, as would be expected of an inhibitor acting solely on the outside.

It has been found that the suggestion of LeFevre and Marshall (1959) that phlorizin action may be accounted for by the contamination by and/or hydrolysis into phloretin, does not hold true since there is great

similarity in the site of inhibition by phlorizin in cells from both species and that there are great differences in the location of inhibition by phloretin in the two species. The differences in the presumed sides of actions by phloretin and phlorizin in the two species suggests that the binding sites for these inhibitors may be distinct, however the possibility that phloretin and polyphloretin phosphate interact at the same site on the external surface of the erythrocyte membrane is not ruled out.

It had earlier been reported that the glucose moiety on phlorizin does not appear to play any part in the drugs ability to inhibit the hexose transfer system in erythrocytes since its replacement with a methyl group does not alter the potency of inhibition, furthermore phloretin,which lacks the glucose moiety, is at least 100 fold more effective at inhibiting hexose transfer (Rosenberg and Wilbrandt, 1957).

Inhibition of Hexose Transfer with Halo-2,4-dinitrobenzenes

In comparing the inactivation of the hexose transfer system by Fluro-2,4-dinitrobenzene (FDNB) and also bromo-2,4-dinitrobenzene (BrDNB), incubated either in the presence or in the absence of 2-deoxyglucose, in erythrocytes from the fetal and newborn guinea-pigs and adult humans, some interesting observations have been made.

The enhancement of the rate of inhibition, of the hexose transfer by FDNB, with 2-deoxyglucose is most conspicuous in human cells. There is an acceleration of the inhibition rate by a factor of about 2.5 in human cells with this inhibitor while the acceleration in fetal and newborn guinea-pig cells is by a factor of about 1.25 only.

The presence or the absence of any 2-deoxyglucose during the inhibition reaction with BrDNB appears to have no significant effect on the inactivation rate in human cells. The inhibition of the transfer system in fetal and newborn guinea-pig erythrocytes by BrDNB would, however, seem to proceed faster in the absence of 2-deoxyglucose than in its presence.

It has therefore been found that the fetal and newborn guinea-pig and human erythrocytes respond differently to inactivation of the hexose transfer system by FDNB and BrDNB, in the sense that the inactivation reaction is accelerated much more in human cells by the inclusion of 2-deoxyglucose in the incubating medium, than in the former cells. Furthermore the difference between the inactivation by BrDNB in the two species is seen to be in the reverse direction to FDNB inactivation. That is to say that the presence of 2-deoxyglucose during the inactivation reaction with BrDNB has a very small effect on human cells however with fetal and newborn guinea-pig erythrocytes the presence of 2-deoxyglucose would actually seem to protect the hexose transfer system from inactivation.

The enhancement of FDNB inhibition by specific interaction of sugars and reversible inhibitors with the hexose transfer system has been cited as evidence for a conformational change in the carrier macromolecule, associated with sugar transport (Krupka, 1971). Further work with a series of Halo-2,4-dinitrobenzenes led Krupka and Devés (1980) to postulate that the FDNB binding site on the cell membrane was probably involved in the carrier reorientation rather than substrate binding, thus on the basis of this interpretation it would be reasonable to suggest that the differences between fetal and newborn guinea-pig and adult human erythrocytes hexose transfer system may be due to this system in fetal and newborn guinea-pigs being such that the halo-2,4-dinitrobenzene site may not require the degree of exposure, associated with the conformational change during translocation of a sugar molecule, as that required in human cells.

The finding that a transportable sugar interferes with the inactivation of the hexose transfer system by BrDNB in fetal and newborn guinea-pig red cells may indicate that the binding site for this inhibitor may lie close to the hexose binding site, since the bound sugar molecule apparently interferes with the binding of the bulkier BrDNB molecule and not the smaller FDNB molecule.

Further experiments to support this hypothesis are of course necessary. These experiments could conceivably involve the investigation of the effect of the presence or absence of 2-deoxyglucose on the rate of inactivation

of the hexose transfer system by a series of halo-2,4-dinitrobenzenes, for example the fluro-, chloro-, bromo- and the iodo-2,4-dinitrobenzene. In addition to this, the effect of a series of transportable sugars and, possibly some disaccharides, on the rate of inactivation of the hexose transport system by BrDNB may reveal important supportive evidence.

In concluding the discussion on investigations of various drug effects on the hexose transfer system in erythrocytes from fetal and newborn guinea-pigs and adult humans, it is considered worth mentioning some general points that were taken into account at the onset and during the above projects.

All experiments were carried out at 16 ^{o}C and pH 7.4. The reason for working at 16 ^{o}C was that with earlier experiments using ethylidene glucose it was found necessary to use low concentrations. The decrease in the sugar concentration required a decrease in temperature to compensate for the fact that at low concentrations, the sugar fluxes are relatively faster. Another added advantage for working at 16 ^{o}C was that the diffusion of ethylidene glucose into or out of cells during the time required for sampling, was so small that it was considered insignificant.

All experiments were performed at pH 7.4 since any fluctuations in this value would not involve great changes in kinetic parameter of hexose transport in erythrocytes. Also, since the pH dependence of the hexose transfer

system in fetal and newborn guinea-pig red cells is similar to that found in human cells, any systematic errors due to changes in pH would be a minimum.

The use of newborn guinea-pig blood was made, rather than the fetal blood, as it was found that sufficient quantities of red blood cells with high glucose permeability remained in the postnatal circulation, in which the hexose transport kinetic parameters could be measured effectively, thus justifying the use of blood from 1-6 day old guinea-pigs. The use of newborn guinea-pig blood had a two fold advantage. The first being that of elimination of the need to sacrifice all fetuses present in the uterus of the pregnant guinea-pig, together with the sow since on average blood from two newborn guinea-pigs was sufficient for any one experimental run. Secondly, the process whereby blood was obtained from newborn guinea-pigs was relatively much simpler than the process involving anaesthetising the pregnant guinea-pig and obtaining the blood from the umbilical vessels.

Possible Physiological Function of the Hexose Transfer System in Fetal Erythrocytes

That the fast hexose transfer system, which is present in red cells from a number of fetal and newborn mammals, but which is absent, or almost absent, from the adult of the species, is very suggestive of the possibility that this system must have a vital physiological function

at the pre-natal or fetal stage of the animal. What this function might be is very obscure, however Widdas (1980) and Aubby and Widdas (1980) have postulated a possible role for this facilitated hexose transfer system in the fetal erythrocyte.

The facilitated transfer system in fetal cells suggests that it may have a role in hexose transfer from the maternal to the fetal circulation, since the hexose transfer across the red cell membrane occurs rapidly and the carriage in these cells would provide an additional capacity for transport, over and above that of the fetal plasma.

On the basis of simple kinetics described by Baker and Widdas (1973) and parameters appropriate to fetal and newborn guinea-pig cells (Aubby and Widdas, 1980), calculations, on a programmable calculator, were made to see what, if any, additional advantage would be provided by red cells with a rapid hexose transfer system. The assumptions that were made during these calculations were, that the movement of glucose from the tissue to the blood plasma was by simple diffusion and that this was rapid and able to equilibrate in about one second, and, secondly, that the process of facilitated transfer was responsible for the equilibration of glucose between the plasma and the red cells. All calculations were made at low glucose concentrations and a hematocrit of 45%, values which are more likely to be found <u>in vivo</u>.

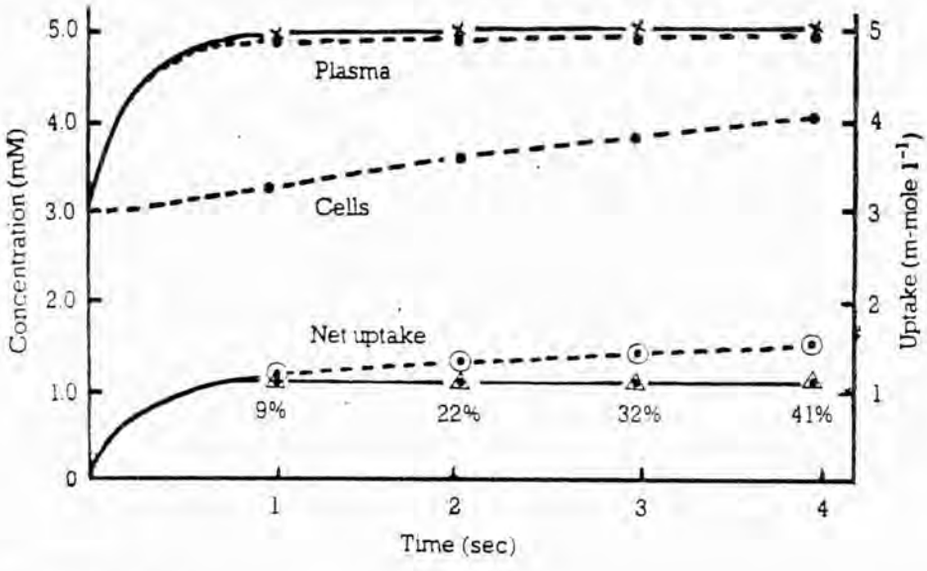


Figure 8.1

Results from model calculations to show the concentration changes and uptake of glucose by blood initially equilibrated at 3 mM while in a capillary where the tissue glucose concentration is 5 mM. Points × and continuous lines and points • and interrupted lines represent the rise in plasma concentration with time in the absence and the presence of glucose permeable cells. The middle line represents the rise in concentration of red cell whose permeability was similar to fetal guinea-pig red cells at 37 °C. The lower lines and right hand scale represents the net uptake of glucose if carried by plasma alone (continuous line) and if carried by both both cells and plasma (interrupted line). The increment due to sugar carried in the red cells is indicated as a percentage at each sec. This figure is typical of results with other starting glucose concentrations.

The results of such calculations are shown in Figure 8.1, where the capillary bed is assumed to be held at a constant concentration of 5 mM glucose and the blood entering such a capillary bed is at a lower concentration of 3 mM glucose. These results are typical of several similar calculations, but with different starting concentrations of glucose.

From these calculations, it can be seen that the plasma concentration changes very rapidly and is at equilibrium value in about one second. The uptake of glucose by red cells is not very significant in this time. If, however, it was assumed that the blood remained in the capillary bed for two or more seconds, then a significant amount of glucose would be taken up by the red cells. Thus blood with cells possessing this facilitated hexose transfer would be able to carry glucose over and above that of a blood in which the cells did not possess a fast hexose transfer system and in which the glucose would be carried only in the plasma.

The calculations showed that over a period of one second, the cells with a facilitated transfer system would enable an extra 9% uptake of glucose by the blood. As indicated above, over a period of two seconds this excess glucose carriage would be increased significantly to around 22%, with further increases of up to 32% and 41%, which would occur if the blood remained in this capillary bed for a period of around three and four seconds respectively. With a higher hematocrit one may envisage that the cells

with such a facilitated transfer system would provide the fetal blood an even greater advantage in taking up glucose.

As a consequence of this , these cells would have a tendency to lower the plasma concentration. The equilibrium of sugar between the cells, which initially were unable to take up significant amounts of glucose, and the plasma, which took up glucose rapidly, would be the cause for this change in plasma concentration. The subsequent loss of glucose from these cells would help maintain a high concentration in the plasma as sugar is diffusing into tissue spaces.

Thus for human and other primates, a facilitated transfer system in both fetal and adult blood cells, could provide a twofold advantage to the animal in the sense that, as the glucose concentration of the maternal plasma, in the placenta, is reduced by its movement into the fetal circulation, the maternal red blood cells would release their supply of glucose into the plasma thus maintaining a higher plasma concentration on the maternal side. The facilitated transfer system in fetal red blood cells would, as discussed above, enable greater quantities of sugar to be taken up on the fetal side. The non-primate fetal red cells, with this facilitated transfer system only in the fetus could therefore be seen as providing an additional and advantageous capacity for the carriage of glucose from the placenta to the fetal tissue only.

It follows, therefore, that the persistence of the facilitated transfer system in adult human and other primate

erythrocytes may thus be a peculiarity of their evolution, since this system in fetal cells for most non-primates is seen to serve a physiological function only at the fetal stage. Thus the remarkable stability or rather the lack of gross abnormalities or the anomalous absence of the hexose trnasfer system in humans supports the contention that this system is either genetically very stable or else any abnormality which may occur does so with fatal consequences, at a very early stage of development (Widdas, 1980).

Possible Future Investigations

In addition to the two possible lines of investigations already mentioned in this chapter, namely with Cytochalasin B and the halo-2,4-dinitrobenzenes, some other possibilities are discussed below. It is emphasised that these possibilities are discussed very superficially and that a great deal of supportive evidence has not been submitted.

The fetal and newborn guinea-pig, or for that matter adult human red blood cells, do not constitute a homogeneous cell population and phenylhydrazine-induced reticulocytes in adult pigs possess a fast hexose transfer system not unlike that found in newborn piglet red blood cells (Zeidler et al., 1976). It is noted that the hexose transfer capability of naturally occurring reticulocytes from newborn piglets is reduced to a level similar to that found in phenylhydrazine-induced reticulocytes in adult pigs, furthermore that these reticulocytes in adult pigs

lose their hexose transfer ability in the course of maturation (Kim and Luthra, 1977). This suggests that red blood cells at different stages in maturation may possess different degrees of facilitation of hexose transport thus on the basis of above, it would seem interesting to investigate the way in which the hexose transfer system varies, if at all, between the reticulocytes and mature red cells in the adult human and also compare this with reticulocytes and mature neonatal red blood cells.

There has been some suggestion that hemoglobin binds to band 3 of the erythrocyte membrane, which is postulated to contain the membrane component responsible for the facilitated hexose transport in erythrocytes (Trosper and Levy, 1977; Phutakul and Jones, 1979; Mullins and Langdon, 1980) and also that hemoglobin may have a role in accounting for some of the experimental observations with the hexose transfer system in erythrocytes (Naftalin and Holman, 1977). This suggests another avenue for future projects. It is possible to conceive a project whereby the effect of different concentrations of hemoglobin, contained in resealed erythrocyte ghosts, are investigated and the kinetic parameters of hexose transport studied to provide information on the subject. Another relatively simple procedure for examining the effects of hemoglobin on the hexose transfer system in erythrocytes would be to use inside-out erythrocyte ghosts and study the effect of different concentrations of hemoglobin on these preparations. However the

measurement of hexose fluxes in such preparations is still not very satisfactory and some further work would need to be done on the preparations themselves.

It has been shown that high voltages applied for very short durations, across the erythrocyte membrane, punch holes in the cell membrane and the size of these holes may be predetermined by varying the duration and/or the voltage of the pulse. Subsequent incubation of cells treated in this manner, at 37 ^{o}C and at isotonicity leads to the resealing of these holes, such that these cells are almost indistinguishable from normal cells (Kinosita and Tsong, 1977). Thus the possibility of using such preparations is of obvious interest. These preparations may for instance allow the cells to be loaded with a disaccharide and resealed, hence enabling the competition of a disaccharide, which may either be present solely on the inside or on the outside to be studied. The comparison of such competitions with drugs which do not normally enter the cell could also be observed.

Several studies have tended to reveal the asymmetrical nature of the hexose transport system in erythrocytes, and speculations on the consequences and physiological implications of such systems have been made. It is however, evident that only future works investigating the fuller implications and consequences of facilitated hexose transfer in erythrocytes and/or from cells in other tissues would provide a clearer understanding.

Comparative studies offer opportunities of investigating the affinities and rates of action of the hexose transfer system for a variety of sugars and inhibitors, but opportunities are rarely present for studying these effects in cells from several species. However, previous works by various authors show that work with red cells from fetal or newborn animals would be feasable, at least for rabbit (Augustin et al., 1967), dog (Lee et al., 1976), pig (Zeidler et al., 1976) and guinea-pig (Dawson and Widdas, 1964; Aubby and Widdas, 1979). Laboratories with such a wide choice of species are few, but not impossible, however cost would be an obvious problem. Extension of the Cytochalasin B studies to these species together with measurements of exchange flux rates would enable a comparitive study to be made of the number of Cytochalasin B binding sites per cell and also an estimation of the turnover numbers for the facilitated transfer system.

SUMMARY

Summary

1. The kinetic parameters of hexose exchange in fetal and newborn guinea-pig erythrocyte have been measured at 16 ^{o}C.

2. The asymmetry of the hexose transfer in fetal and newborn guinea-pig erythrocytes towards non-transportable inhibitors has been studied and was shown to be similar, but of a lesser degree, than that found in human red cells.

3. A marked asymmetry of affinities was exhibited by Cytochalasin B, between the inside and the outside hexose transfer sites in fetal and newborn guinea-pig erythrocytes. The affinity of Cytochalasin B for fetal and newborn guinea-pig cells was less than that for human cells.

4. The binding of Cytochalasin B to fetal and newborn guinea-pig erythrocyte membranes has been studied. There were fewer hexose transfer components, with faster turnover rates, in these cell membranes compared with human cells.

5. The inhibition of the hexose transfer system produced by phloretin, phlorizin and polyphloretin phosphate was qualitatively similar in cells from fetal and newborn guinea-pigs and humans, but the inhibitory affinities were different in the two species.

6. The change in the rates of inhibition, in the presence of 2-deoxyglucose, by FDNB and BrDNB was different in erythrocytes from fetal and newborn guinea-pigs and adult humans.

7. The possible physiological function of the facilitated transfer of sugars in fetal cells has been discussed.

8. Some possible avenues of future research on the hexose transport mechanism have been suggested.

REFERENCES

References

ALLISON, A.C., DAVIES, P. & DePETRIS, S. (1971) Role of contractile microfilaments in macrophage movement and endocytosis. Nature New Biol. 232, 153-155.

ALVARADO, F. (1967) Hypothesis for the interaction of phlorizin and phloretin with membrane carriers for sugars. Biochim. Biophys. Acta 135, 483-495.

AUBBY, D.S. & WIDDAS, W.F. (1979) Asymmetry in the hexose transfer system of erythrocytes from newborn guinea-pigs. J. Physiol. 293, 73P.

AUBBY, D.S. & WIDDAS, W.F. (1980) Asymmetry of hexose transfer system in erythrocytes of fetal and newborn guinea-pigs. J. Physiol. 309, 317-327.

AUGUSTIN, H.W., ROHDEN, L.V. & HACKER, M.R. (1967) Uber einige eigenschaften des monosaccharid transport systems in erythrozyten neuge borenen und envashsener kaninchen. Acta Biol. Med. Ger. 19, 723-735.

BAKER, G.F., BASKETTER, D.A. & WIDDAS, W.F. (1978) Asymmetry of the hexose transfer system in human erythrocytes. Experiments with non-transportable inhibitors. J. Physiol. 278, 377-388.

BAKER, G.F. & ROGERS, H.J. (1973) Further effects of chlorpromazine on the hexose permeability of the human erythrocyte. J. Physiol. 232, 597-608.

BAKER, G.F. & WIDDAS, W.F. (1972) A glucose derivative which enters the human red cell by simple diffusion. J. Physiol. 221, 10P-11P.

BAKER, G.F & WIDDAS, W.F. (1973a) The permeation of human red cells by 4,6-O-ethylidene-α-D-gluco-pyranose (ethylidene glucose). J. Physiol. 231, 129-142.

BAKER, G.F. & WIDDAS, W.F. (1973b) The asymmetry of the facilitated transfer system for hexose in human red cells and the simple kinetics of a two component model. J. Physiol. 231, 143-165.

BARNETT, J.E.G., HOLMAN, G.D., CHALKLEY, R.A. & MUNDAY, K.A. (1975) Evidence for two asymmetric conformational states in the human erythrocyte sugar-transport system. Biochem. J. 145, 417-429.

BARNETT, J.E.G., HOLMAN, G.D. & MUNDAY, K.A. (1973) An explanation of the asymmetry binding of sugars to the human erythrocyte sugar-transport system. Biochem. J. 135, 539-541.

BASKETTER, D.A. & WIDDAS, W.F. (1977) Competitive inhibition of hexose transfer in human erythrocytes by Cytochalasin B. J. Physiol. 265, 39P-40P.

BASKETTER, D.A. & WIDDAS, W.F. (1978) Asymmetry of the hexose transfer system in human erythrocytes. Comparison of the effects of Cytochalasin B, phloretin and maltose as competitive inhibitors. J. Physiol. 278, 389-401.

BATT, E.R., ABBOTT, R.E. & SCHACHTER, D. (1976) Impermeant maleimides. Identification of an exofacial component of the human erythrocyte hexose transport mechanism. J. Biol. Chem. 251, 7184-7190.

BATT, E.R. & SCHACHTER, D. (1973) Transport of monosaccharides I. Asymmetry in the human erythrocyte mechanism. J. Clin. Invest. 52, 1686-1697.

BENEŠ. I, KOLINSKA, J. & KOTYK, A. (1972) Effect of phloretin on monosaccharide transport in erythrocyte ghosts. J. Memb. Biol. 8, 303-309.

BIHLER, I., CAVERT, H.M. & FISHER, R.B. (1965) A differential effect of inhibitors on sugar penetration into the isolated rabbit heart. J. Physiol. 180, 168-177.

BLOCH, R. (1973) Inhibition of glucose transport in the human erythrocyte by Cytochalasin B. Biochemistry 12, 4799-4801.

BLOOM, W. (1938) In "Hand Book of Hematology" (ed. Downey, H.) Vol. 2, pp 863-922. New York: Hoeber.

BOWYER, F. & WIDDAS, W.F. (1955) Erythrocyte permeability to erythritol. J. Physiol. 129, 7P-8P.

BOWYER, F. & WIDDAS, W.F. (1956) Inhibition of the hexose transfer system in erythrocytes. Abstr. Commun. XX Intern. Physiol. Congr., pp 122-213.

BOWYER, F. & WIDDAS, W.F. (1958) The action of inhibitors on the facilitated hexose transfer system in erythrocytes. J. Physiol. 141, 219-232.

CHAN, S.S. & LOTSPEICH, W.D. (1962) Comparative effect of phlorizin and phloretin on glucose transport in the cat kidney. Amer. J. Physiol. 203, 975.

CHEN, L.M. & LeFEVRE, P.G. (1965) Disaccharide specificity of human red cell glucose transport system. Fed. Proc. 24, 465.

CHESNEY, R., SACKTOR, B. & KLEINZELLER, A. (1974) The binding of phlorizin to the isolated luminal membrane of the renal proximal tubule. Biochem. Biophys. Acta 332, 263-277.

CHEUNG, J.F., WHITFIELD, C.F., REGEN, D.M. & MORGAN, H.E. (1976) A kinetic study of the monosaccharide transport system in avian erythrocytes. Fed. Proc. 35, 780.

CRANE, R.K. (1960) Intestinal absorption of sugars. Physiol. Rev. 40, 789-825.

CZECH, M.P., LYNN, D.G. & LYNN, W.S. (1973) Phlorizin-receptor interactions in fat cell plasma membrane. B.B.A. 323, 639-642.

DANIELLI, J.F. (1954) The present position in the field of facilitated diffusion and selective active transport. Proc. Symp. Colston Res. Soc. 7, 1.

DAWSON, A.C. & WIDDAS, W.F. (1964) Variation with temperature and pH of the parameters of glucose transfer across the erythrocyte membrane in the foetal guinea-pig. J. Physiol. 172, 107-122.

DEVÉS, R. & KRUPKA, R.M. (1978a) A new approach in the kinetics of biological transport. The potential of reversible inhibition studies. Biochim. Biophys. Acta 510, 186-200.

DEVÉS, R. & KRUPKA, R.M. (1978b) Cytochalasin B and the kinetics of inhibition of biological transport. A case of asymmetric binding to the glucose carrier. Biochim. Biophys. Acta 510, 339-348.

DIXON, M. & WEBB, E.C. (1964) Enzymes. London: Longmans

EILAM, Y. (1975) Two-carrier models for mediated transport II. Glucose and galactose equilibrium exchange experiments in human erythrocytes as a test for several two-carrier models. Biochim. Biophys. Acta 401, 364-369.

EILAM, Y. & STEIN, W.D. (1972) A simple resolution of the kinetic anomaly in the exchange of different sugars across the membrane of the human red blood cell. Biochim. Biophys. Acta 266, 161-173.

ESTENSEN, R.D. & PLAGEMANN, P.G.W. (1972) Cytochalasin B: Inhibition of glucose and glucosamine transport. Proc. Natl. Acad. Sci. (U.S.) 69, 1430-1434.

FORSLING, M.L. & WIDDAS, W.F. (1968) The effect of temperature on the competitive inhibition of glucose transfer in human erythrocytes by phenolphthalein, phloretin and stilboestrol. J. Physiol. 194, 545-554.

FRASH, W., FROHNERT, P.P., BODE, F., BAUMANN, K. & KINNE, R. (1970) Competitive inhibition of phlorizin binding by D-glucose and the influence of sodium: A study on isolated brush border membrane of rat kidney. Pflügers Arch. 320, 265-284.

GARDOS, G. (1954) Akkumulation der kaliamionen durch Menschliche Blutkörperchen. Acta Physiol. Hung. 6, 191-199.

GECK, P. (1971) Eigenschaften eines asymmetrischen carrier-modells für den Zuckertransport am Menschlichen Erythrozyten. Biochim. Biophys. Acta 241, 462-472.

GLOSSMAN, H. & NEVILLE Jr, D.M. (1972) Phlorizin receptors in isolated kidney brush border membranes. J. Biol. Chem. 247, 7779-7789.

GOLDACRE, R.J. (1958) Surface films, their collapse and compression, the shape and size of cells and the origins of life. In "Surface Phenomena in Chemistry and Biology" (eds Danielli, J.F., Pankhurst, K.G.A. & Riddiford, A.C.) Oxford: Pergamon Press.

GOMPERTS, B.D. (1977) In "The Plasma Membrane. Models for Structure and Function" by Gomperts, B.D. New York, London: Academic Press.

GRYNS, G. (1896) Über den Einfluss geloster stoffe auf die roten Blutzellen inverbindung mit den Erschunungen der Osmose und Diffusion. Pflüg. Arch. ges. Physiol. 163, 86-119.

GUIDOTTI, G. (1972) The composition of biological membranes. Arch. Intern. Med. 129, 194-201.

HANES, C.S. (1932) Studies on plant amylases. I. The effect of starch concentration upon the velocity of hydrolysis by the amylase of germinating barley. Biochem. J. 26, 1406-1421.

HELMREICH, E. & CORI, C.F. (1957) Studies of tissue permeability. II. The distribution of pentoses between plasma and muscle. J. Biol. Chem. 224, 663-673.

HÖBER, R. & ØRSKOV, S.L. (1933) Untersuchungen über die Permeiegeschwindigkeit von Anelektrolyten bei den roten Blutkörperchen verschiedener Tierarten. Pflügers Archiv. 231, 599-615.

HOFFMAN, J.F. (1958) Physiological characteristics of human red blood cell ghosts. J. Gen. Physiol. 42, 9-28.

HOLMAN, G.D. (1980) An allosteric pore model for sugar transport in human erythrocytes. Biochim. Biophys. Acta 599, 202-213.

HUGGETT, A.St.G. & WIDDAS, W.F. (1951) The relationship between mammalian foetal weight and conception age. J. Physiol. 114, 306-317.

JACOBS, M.H., GLASSMAN, H.N. & PARPART, A.K. (1935) Osmotic properties of the erythrocyte. VII. The temperature coefficients of certain hemolytic processes. J. Cell Comp. Physiol. 7, 197-225.

JACOBS, M.H., PARPART, A.K. & CARSON, S.A. (1937) Osmotic properties of erythrocytes. The effect of low concentrations of electrolytes on hemolysis by penetrating non-electrolytes and on cell volume. J. Cell Comp. Physiol. 9, 177-190.

JARVIS, S.M. & YOUNG, J.D. (1982) Nucleoside translocation in sheep reticulocytes and fetal erythrocytes: A proposed model for the nucleoside transporter. J. Physiol. 324, 47-66.

JENNINGS, M.L. & SOLOMON, A.K. (1976) Interaction between phloretin and the red blood cell membrane. J. Gen. Physiol. 67, 381-397.

JUNG, C.Y. (1975) Carrier-mediated glucose transport across human red cell membranes. In "The Red Blood Cell" (ed. Surgenor, D.M.) 2nd ed., pp 705-749. New York: Academic Press.

JUNG, C.Y., PINKOFSKY, H.B. & COWDEN, M.W. (1980) Cytochalasin B-binding proteins in rabbit erythrocyte membranes and their post-natal changes in relation to the glucose carrier activity. Biochim. Biophys. Acta 597, 145-154.

JUNG, C.Y. & RAMPAL, A.L. (1975) Effect of Cytochalasin B on equilibrium exchange flux of D-glucose across human erythrocyte membranes. Fed. Proc. Fed. Am. Soc. Exp. Biol. 34, 238.

JUNG, C.Y. & RAMPAL, A.L. (1976) Cytochalasin B binding proteins and glucose carriers in human erythrocyte ghosts. Fedn. Proc. 35, 780.

KABACK, H.R. (1970) Transport. Annu. Rev. Biochem. 39, 561-598.

KAHLENBERG, A., URMAN, B. & DOLANSKY, D. (1971) Preferential uptake of D-glucose by isolated human erythrocyte membranes. Biochemistry, N.Y. 10, 3154-3162.

KALCHAR, H. (1936) Inhibitory effect of phloridzin and phloretin on kidney phosphatase. Nature 138, 289.

KARLISH, S.J.D., LIEB, W.R., RAM, D. & STEIN, W.D. (1972) Kinetic parameters of glucose efflux from human red blood cells under zero-*trans* conditions. Biochim. Biophys. Acta 255, 126-132.

KIM, H.D. & LUTHRA, M.G. (1977) Pig reticulocytes. III. Glucose permeability in naturally occurring reticulocytes and red cells from newborn piglets. J. Gen. Physiol. 70, 171-185

KINOSITA, K. Jr & TSONG, T.Y. (1977) Voltage-induced pore formation and hemolysis of human erythrocytes. Biochim. Biophys. Acta 471, 227-242.

KIPNIS, D.M. & CORI, C.F. (1957) Studies of tissue permeability. III. The effect of insulin on pentose uptake by the diaphragm. J. Biol. Chem. 224, 681-693.

KLETZIEN, R.F. & PURDUE, J.F. (1973) The inhibition of sugar transport in chick embryo fibroblasts by Cytochalasin B. Evidence for a membrane-specific effect. J. Biol. Chem. 248, 711-719.

KLETZIEN, R.F. & PURDUE, J.F. (1974) The mechanism by which Cytochalasin B, phloretin and diethylstilbestrol inhibit sugar transport in cultured cells. J. of Cell Biol. 63, 171a.

KLETZIEN, R.F., PURDUE, J.F. & SPRINGER, A. (1972) Cytochalasin A and B. Inhibition of sugar uptake in cultured cells. J. Biol. Chem. 247, 2964-2966.

KOTYK, A. & HÖFER, M. (1965) Uphill transport of sugars in the yeast Rhodotorula Gracilis. Biochim. Biophys. Acta 102, 410-422.

KOTYK, A., KOLINSKA, J., VERÉS, K. & SZAMMER, J. (1965) Inhibition by phloretin and phlorizin derivatives of sugar transport in different cells. Biochem. Z. 342 (1965), 129-138.

KOZAWA, S. (1914) Beiträge zum arteigenen verhalten der roten Blutkörperchen III. Artdifferenzen in der Durchlässigkeit der roten Blutkörperchen. Biochem. Z. 60, 231-256.

KREGENOV, F.M. (1977) Transport in avian red cells. In "Membrane Transport in Red Cells" (ed. Lew, Ellory) pp 383-426. Academic Press.

KRUPKA, R.M. (1971a) Evidence for a carrier conformational change associated with sugar transport in erythrocytes. Biochemistry 10, 1143-1148.

KRUPKA, R.M. (1971b) Inhibition of sugar transport in erythrocytes by fluorodinitrobenzene. Biochemistry 10, 1148-1153.

KRUPKA, R.M. & DEVÉS, R. (1980) Reaction of the glucose carrier in erythrocytes with halodinitrobenzenes. J. Biol. Chem. 255, 2051-2055.

LACKO, L. & BURGER, M. (1962) Interaction of some disaccharides with the carrier system for aldoses in erythrocytes. Biochem. J. 83, 622-625.

LACKO, L, WITTKE, B. & GECK, P. (1972) The pH dependence of exchange transport of glucose in human erythrocytes. J. Cell Physiol. 80, 73-78.

LEE, P., AUVIL, J., GREY, J.E. & SMITH, M. (1976) 3-O-methyl glucose transport in newborn and adult dog red cells. Fed. Proc. Fed. Am. Soc. Exp. Biol. 35, 780.

LeFEVRE, P.G. (1948) Evidence of active transfer of certain non-electrolytes across the human red cell membrane. J. Gen. Physiol. 31, 505-527.

LeFEVRE, P.G. (1954) The evidence for active transport of monosaccharides across the red cell membrane. Symp. Soc. Exp. Biol. 8, 118-135.

LeFEVRE, P.G. (1959) Molecular structural factors in competitive inhibition of sugar transport. Science 130, 104-105.

LeFEVRE, P.G. (1961) Sugar transport in the red blood cells: structure-activity relationship in substrates and antagonists. Pharmacol. Rev. 13, 39-70.

LeFEVRE, P.G. (1962) Rate and affinity in human red blood cell sugar transport. Amer. J. Physiol. 203, 286-290.

LeFEVRE, P.G. (1972) In "Metabolic Transport" (ed. Hokin, L.E.) p 385. New York: Academic Press.

LeFEVRE, P.G. (1973) A model for erythrocyte sugar transport based on substrate-conditioned "introversion" of binding sites. J. Membrane Biol. 11, 1-19.

LeFEVRE, P.G. (1975a) Present state of the carrier hypothesis. Cur. Top. Membr. Transp. 7, 109-215.

LeFEVRE, P.G. (1975b) A comparison of recent suggestions for the functional organisation of red cell sugar transport sites based on kinetic observations. Annals - New York Academy of Science 264, 398-413.

LeFEVRE, P.G. & LeFEVRE, M.E. (1952) The mechanism of glucose transfer into and out of human red cells. J. Gen. Physiol. 35, 891-906.

LeFEVRE, P.G. & MARSHALL, J.K. (1958) Conformational specificity in biological sugar transport system. Amer. J. Physiol. 194, 333-337.

LeFEVRE, P.G. & MARSHALL, J.K. (1959) The attachment of phloretin and analogues to human erythrocytes in connection with inhibition of sugar transport. J. Biol. Chem. 234, 3022-3026.

LEPKE, S. & PASSOW, H. (1973) Asymmetric inhibition by phlorizin of sulfate movements across the red blood cell membrane. Biochim. Biophys. Acta 298, 529-533.

LEVINE, M., OXENDER, D.L. & STEIN, W.D. (1965) The substrate-facilitated transport of the glucose carrier across the human erythrocyte membrane. Biochim. Biophys. Acta 109, 151-163.

LEVINE, R., GOLDSTEIN, M.S., KLEIN, S.P. & HUDDLESTUN, B. (1949) The action of insulin on the distribution of galactose in eviscerated nephrectomized dogs. J. Biol. Chem. 179, 985-986.

LEVINE, S., FRANKI, N. & HAYS, R.M. (1973) Effect of phloretin on water and solute movement in toad bladder. J. Clin. Invest. 52, 1435-1442.

LIEB, W.R. & STEIN, W.D. (1970) Quantitative predictions of a noncarrier model for glucose transport across the human red cell membrane. Biophys. J. 10, 585-609.

LIEB, W.R. & STEIN, W.D. (1971) New theory for glucose transport across membranes. Nature New Biol. 230, 108-109.

LIEB, W.R. & STEIN, W.D. (1972a) Carrier and non-carrier models for sugar transport in the human red blood cell. Biochem. Biophys. Acta 265, 187-207.

LIEB, W.R. & STEIN, W.D. (1972b) The influence of unstirred layers on the kinetics of carrier-mediated transport. J. Theor. Biol. 36, 641-645.

LIN, E.C.C. (1971) In "Structure and Function of Biological Membranes" (ed. Rothfield, L.I.) p 286. New York: Academic Press.

LIN, S., SANTI, D.V. & SPUDICH, J.A. (1974) Biochemical studies on the mode of action of Cytochalasin B. Preparation of [^{3}H] Cytochalasin B and studies on its binding to cells. J. Biol. Chem. 249, 2268-2274.

LIN, S. & SPUDICH, J.A. (1974) Biochemical studies on the model of action of Cytochalasin B binding to red cell membrane in relation to glucose transport. J. Biol. Chem. 249, 5778-5783.

MACEY, R.I. & FARMER, R.E.L. (1970) Inhibition of water and solute permeability in human red cells. Biochim. Biophys. Acta 211, 104-106.

MAJNO, G., BOUVIER, C.A., GABBIANI, G., RYAN, C.B. & STATKOV, P. (1972) Kymographic recording of clot retraction. Effect of papaverine, theophylline and Cytochalasin B. Thrombos. Diathes Haemorrh. 28, 49-53.

MASIAK, S.J. & LeFEVRE, P.G. (1972) Failure of equilibrium dialysis to show selective monosaccharide binding by erythrocyte membranes. J. Membrane Biol. 9, 291-296.

MASIAK, S.J. & LeFEVRE, P.G. (1977) Glucose transport inhibition by proteolytic degradation of human erythrocyte membrane inner surface. Biochim. Biophys. Acta 465, 371-377.

MAWE, R.C. & HEMPLING, H.G. (1965) The exchange of C^{14} glucose across the membrane of the human erythrocyte. J. Cell.Comp. Physiol. 66, 95-104.

MILLER, D.M. (1965) The kinetics of selective biological transport. II. Equations for induced uphill transport of sugars in human erythrocytes. Biophys. J. 5, 417-423.

MILLER, D.M. (1968a) The kinetics of selective biological transport. III. Erythrocyte-monosaccharide transport data. Biophys. J. 8, 1329-1338.

MILLER, D.M. (1968b) The kinetics of selective biological transport. IV. Assessment of three carrier systems using the erythrocyte-monosaccharide transport data. Biophys. J. 8, 1339-1352.

MILLER, D.M. (1969) In "Red Cell Membrane Structure and Function" (eds, Jamieson, G.A. & Greenwalt, T.J.) p 240. Philadelphia, Pennsylvania: Lippincott.

MILLER, D.M. (1972) The effect of unstirred layers on the measurement of transport rates in individual cells. Biochim. Biophys. Acta 266, 85-90.

MIZEL, S.B. (1973) Differential effect of Cytochalasin B on the two possible modes of 2-deoxyglucose transport in HeLa cells. Nat. New. Biol. 243, 125-126.

MIZEL, S.B. & WILSON, L. (1972) Inhibition of the transport of several hexose in mammalian cells by Cytochalasin B. J. Biol. Chem. 247, 4102-4105.

MOND, R. (1930) Weitere Vergleichende Untersuchungen über Membranstruktur und Permeabitität der roten Blutkörperchen. Bermerkungen zur Frage de Traubenzuckerpermeabitität. Pflügers Archiv. 224, 161-166.

MULLINS, R.E. & LANGDON, R.G. (1980) Maltosyl isothiocyanate: An affinity label for the glucose transport of the human erythrocyte membrane. I. Inhibition of glucose transport. Biochem. 19, 1199-1205.

McCARTHY, E.F. (1933) A comparison of foetal and maternal haemoglobins in the goat. J. Physiol. 80, 206-212.

NAFTALIN, R.J. (1970) A model for sugar transport across red cell membranes without carriers. Biochim. Biophys. Acta 211, 65-78.

NAFTALIN, R.J. & HOLMAN, G.D. (1977) Transport of sugars in human red cells. In "Membrane Transport in Red Cells" (eds, Ellory, J.D. & Lew, V.L.) pp 257-300. New York: Academic Press.

ØRSKOV, S.L. (1935) Eine Methode zur Fortlaufenden photographischen Aufzeichnung von vokimänderungen der roten Blutkörperchen. Biochem. Z. 279, 241-249.

OWEN, J.D., STEGGALL, M. & EYRING, E.M. (1974) The effect of phloretin on red cell nonelectrolyte permeability. J. Membr. Biol. 19, 79-92.

OXENDER, D.L. (1972) Membrane Transport. Annu. Rev. Biochem. 41, 777-814.

PARK, C.R., CROFFORD, O.B. & KONO, T. (1968) Mediated (nonactive) transport of glucose in mammalian cells and its regulation. J. Gen. Physiol. 52, 2965-3185.

PARK, C.R., POST, R.I., KALMAN, C.F., WRIGHT, J.H., Jr, JOHNSON, L.H. & MORGAN, H.E. (1956) The transport of glucose and other sugars across cell membranes and the effects of insulin. Ciba Fdn Colloq. Endocr. 9, 240-260.

PASSOW, H. (1964) Ion and water permeability of the red blood cell. In "The Red Blood Cell". (ed. Bishop,C. & Surgenor, D.M.) pp 71-139. New York & London:Academic Press.

PENNEL, R.B. (1964) Composition of normal human red cells. In "The Red Blood Cell" (ed. Bishop, C. & Surgenor, D.M.) pp 29-67. New York & London: Academic Press.

PHUTRAKUL, S. & JONES, M.N. (1979) The permeability of bilayer lipid membranes on the incorporation of erythrocyte membrane extracts and the indentification of the monosaccharide transport protein. Biochim. Biophys. Acta 550, 188-200.

PLAGEMANN, P.G.W. & ERBE, J. (1975) Temperature-dependent changes in activation energies of the transport system for nucleosides, choline and deoxyglucose of cultured Novikoff rat hepatoma cells and effects of Cytochalasin B and lipid solvents. J. Membrane Biol. 25, 281-396.

PLAGEMANN, P.G.W. & WOHLHUETER, R.M. (1980) Permeation of nucleosides, nucleic acid bases, and nucleotides in animal cells. Current Topics in Memb. and Transport 14, 225-330.

PONDER, E. (1966) The cell membrane and its properties. In "The Cell" (eds, Brochet, J. & Mirsky, A.E.) Volume II. pp 1-80. New York and London:Academic Press.

POULSSON, L.T. (1930) On the mechanism of sugar elimination in phlorrhizin glycosuria. A contribution to the filtration-reabsorption theory on kidney function. J. Physiol. 69, 411-422.

RÄIHÄ, N. (1958) On the placental transfer of vitamin C. Acta Physiol. Scand. 45, Supp No 155.

REGEN, D.M. & MORGAN, H.E. (1964) Studies of the glucose-transport system in the rabbit erythrocyte. Biochim. Biophys. Acta 79, 151-166.

REGEN, D.M. & TARPLEY, H.L. (1974) Anomalous transport kinetics and the glucose carrier hypothesis. Biochim. Biophys. Acta 339, 218-233.

ROBERTSON, J.D. (1964) In "Cellular Membranes in Development" (ed. Locke, Michael) pp 1-79. New York: Academic Press.

ROSENBERG, S.A. & GUIDOTTI, G. (1968) The protein of human erythrocyte membranes. I. Preparation, solubilization and partial characterization. J. Biol. Chem. 243, 1985

ROSENBERG, T. & WILBRANDT, W. (1962) The kinetics of carrier transport inhibition. Exp. Cell. Res. 27, 100-117.

SCHNELL, K.F., GERHARDT, S., LEPKE, S. & PASSOW, H. (1973) Asymmetric inhibition by phlorizin of halide movement across the red blood cell membrane. Biochim. Biophys. Acta 318, 474-477.

SEN, A.K. & WIDDAS, W.F. (1962a) Determination of the temperature and pH dependence of glucose transfer across the human erythrocyte membrane measured by glucose exit. J. Physiol. 160, 392-403.

SEN, A.K. & WIDDAS, W.F. (1962b) Variations of the parameters of glucose transfer across human erythrocyte in the presence of inhibitors of transfer. J. Physiol. 160, 404-416.

SHEPRO, D, BELAMARICH, F.A., ROBBLEE, L. & CHAO, F.C. (1970) Antimotility effect of Cytochalasin B observed in mammalian clot retraction. J. Cell Biol. 47, 544-547.

SINGER, S.J. & NICOLSON, G.L. (1972) The fluid mosaic model of the structure of cell membranes. Science 175, 720-731.

SJÖLIN, S. (1954) The resistance of red cells in vitro. Acta Paediat. Stockh. 43, Suppl. 98, 1-92.

STADIE, W.C. (1954) Current concepts of the action of insulin. Physiol. Rev. 34, 52-100.

STECK, T.L., FAIRBANKS, G. & WALLACH, D.F.H. (1971) Disposition of the major proteins in the isolated erythrocyte membrane. Proteolytic dissection. Biochem. 10, 2617-2624.

STEIN, W.D. (1967)"The movement of molecules across membranes" by Stein, W.D. New York and London: Academic Press.

STEIN, W.D. (1968) Transport of sugars. Brit. Med. Bull. 24, 146-149.

STIRLING, C.E. (1961) High-resolution radioautography of phlorizin-^{3}H in rings of hamster intestine. J. Cell Biol. 35, 605.

TAVERNA, R.D. & LANGDON, R.G. (1973a) Reversible association of Cytochalasin B with the human erythrocyte membrane. Inhibition of glucose transport and the stoichiometry of Cytochalasin binding. Biochim. Biophys. Acta 323, 207-219.

TAVERNA, R.D. & LANGDON, R.G. (1973b) A new method for measuring glucose translocation through biological membranes and its application to human erythrocyte ghosts. Biochim. Biophys. Acta 298, 412-421.

TAYLOR, N.F. & GAGNEJA, G.L. (1975) A model for the mode of action of Cytochalasin B inhibition of D-glucose transport in the human erythrocyte. Can. J. Biochem. 53, 1078-1084.

THOMAS, D. des S., LUTZAC, M. & MANAVATHA, E. (1974) Cytochalasin selectively inhibits synthesis of a secretory protein, cellulase, in Achlya. Nature 249, 140-142.

TROSPER, T. & LEVY, D. (1977) Photocatalyzed labeling of adipocyte plasma membranes with an aryl azide derivative of glucose. J. Biol. Chem. 252, 181-186.

Van DEENEN, L.L.M. & De GIER, J. (1964) Chemical composition and metabolism of lipids in red cells of various species. In "The Red Blood Cell" (eds, Bishop, C. & Surgenor, D.M.) pp 243-302. New York: Academic Press.

Van STEVENINCK, J. WEED, R.I. & ROTHSTEIN, A. (1965) Localization of erythrocyte membrane sulfhydryl groups essential for glucose transport. J. Gen. Physiol. 48, 617-637.

VINTEN, J., GLIEMANN, J. & ØSTERLIND, K. (1976) Exchange of 3-O-methyl glucose in isolated fat cells. J. Biol. Chem. 251, 794-800.

WESSELL, N.K., SPOONER, B.J., ASH, J.F., BRADLEY, M.D., LUDENS, M.A., TAYLOR, E.L., WREN, J.T. & YAMADA, K.M. (1971) Microfilaments in cellular and development processes. Science 171, 135-143.

WHITESHELL, R.R. & GLIEMANN, J. (1979) Kinetic parameters of transport of 3-O-methyl glucose and glucose in adipocytes. J. Biol. Chem. 254, 5276-5283.

WIDDAS, W.F. (1951) Changing osmotic properties of foetal sheep erythrocytes and their comparison with those of maternal sheep erythrocytes. J. Physiol. 113, 399-411.

WIDDAS, W.F. (1952) Inability of diffusion to account for placental glucose transfer in the sheep and consideration of the kinetics of a possible carrier transfer. J. Physiol. 118, 23-39.

WIDDAS, W.F. (1953) An apparatus for recording erythrocyte volume changes in permeability studies. J. Physiol. 120, 20-21P.

WIDDAS, W.F. (1954) Facilitated transfer of hexoses across the human erythrocyte membrane. J. Physiol. 125, 163-180.

WIDDAS, W.F. (1955) Hexose permeability of foetal erythrocytes. J. Physiol. 127, 318-327.

WIDDAS, W.F. (1974) Pharmacological significance of new concepts for hexose transfers in erythroctyes. In "Drugs and Transport Processes" (ed. Callingham, B.) pp 329-340. New York: Macmillan.

WIDDAS, W.F. (1980) The asymmetry of the hexose transfer system in the human red cell membrane. Curr. Top. Membranes & Transp. 14, pp 165-223. New York and London: Academic Press.

WIETH, J.O., DALMARK, M., GUNN, R.B. & TOSTESON, D.C. (1972) The transfer of monovalent inorganic anions through the red cell membranes. In "Erythrocytes, Thrombocytes, Leucocytes" (eds, Gerlach, E., Moser, K., Deutsch, E. & Wilmanns, W.) pp 71-76 Stuttgart: Georg Thieme Verlag.

WIETH, J.O., FUNDER, J., GUNN, R.B. & BRAHM, J. (1974) Passive transport pathways for chloride and urea through the red cell membrane. In "Comparative Biochemistry and Physiology of Transport" (eds, Bolis, L., Bloch, K., Luria, S.E. & Lynen, F.) pp 317-337. Amsterdam: North-Holland.

WILBRANDT, W. (1947) Die Wirkung des Phlorizins auf die Permeabilität der Menschlichen Erythrocyten für Glukose und Pentosen. Helv. Physiol. Acta 5, C64-C65.

WILBRANDT, W. (1950) Permeabilitätsprobleme. Arct. Exp. Pathol. Pharmakol. 212, 9-29.

WILBRANDT, W. (1954) Secretion and transport of non-electrolytes. Symp. Soc. Exp. Biol. 8, 136-162.

WILBRANDT, W., GUENSBERG, E. & LAUENER, H. (1947) Der Glukoseeintritt durch die Erythrocytenmembran. Helv. Physiol. Acta 5, C20-C22.

WILBRANDT, W. & ROSENBERG, Th. (1957) Strukfurabhängigkeit der Hemmwirkung von Phlorizin und anderen Phloretinderivaten auf den Glukosetransport durch die Erythrocytenmembran. Helv. Physiol. Acta 15, 168-176.

WILBRANDT, W. & ROSENBERG, Th (1961) The concept of carrier transport and its corollaries in pharmacology. Pharmacol. Rev. 13, 109-183.

WILLIAMS, J.A. & WOLFF, J. (1971) Cytochalasin B inhibits thyroid secretion. Biochim. Biophys. Res. Commun. 44, 422-425.

WINDLE, W.F. (1941) The blood of the fetus. In "Physiology of the Fetus" by Windle, W.F. pp 49-58. Philadelphia & London: B. Saunders Co.

WINTROBE, M.M. & SHUMACKER, H.B. Jr. (1936) Erythrocyte studies in the mammalian fetus and newborn. Am. J. Anat. 58, 313-328.

WISE, G.H., CALDWELL, M.J., PARRISH, W.B., FLIPSE, R.J. & HUGHES, J.S. (1947) Changes in cell volume and in concentration of haemoglobin and several inorganic constituents of the blood of calves during early post-natal development. J. Dairy Sci. 30, 983-993.

WOOD, R.E. & MORGAN, H.E. (1969) Regulation of sugar transport in avian erythrocytes. J. Biol. Chem. 244, 1451-1460.

ZIEDLER, R.B., LEE, P. & KIM, H.D. (1976) Kinetics of 3-O-methyl glucose transport in red blood cells of newborn pigs. J. Gen. Physiol. 67, 67-80.

ZIERLER, K.L. (1961) A model of a poorly-permeable membrane as an alternative to the carrier hypothesis of cell membrane penetration. Bull. John Hopkins Hosp. 109, 35-48.

ZIPPER, H. & MAWE, R.C. (1972) The exchange and maximal net flux of glucose across the human erythrocyte.I. The effect of insulin, insulin derivatives and small proteins. Biochim. Biophys. Acta 282, 311-325.

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Asymmetry in the hexose transfer system of erythrocytes from new-born guinea-pigs

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Erythrocytes of fetal and new-born guinea-pigs have a transfer system for hexoses similar in speed to that of human erythrocytes (Widdas, 1955) and using the Sen & Widdas (1962) exit technique have a similar half-saturation constant at 37 and 27 °C but not at lower temperatures (Dawson & Widdas, 1964).

To investigate whether new-born guinea-pig cells have a similar asymmetry to that of human cells we have studied the inhibition of exchange of 3-*O*-methyl glucose at 20 mM and 16 °C by 4,6-*O*-ethylidene-α-D-glucopyranose (ethylidene glucose) and also by methyl-2,3-di-*O*-methyl-α-D-glucopyranoside (trimethyl glucose).

The results were essentially similar to those for human red cells (Baker, Basketter & Widdas, 1978) except that in new-born guinea-pig cells ethylidene glucose had a higher affinity for the inside sites with a K_1 *ca.* 60 mM as opposed to 110 mM for human cells. The outside affinity with an apparent K_i of *ca.* 10 mM was not significantly different from that for human cells (*ca.* 11 mM). The asymmetry in apparent K_is was therefore sixfold in contrast to the tenfold asymmetry for human red cells.

The apparent K_is for trimethyl glucose inhibition were *ca.* 105 mM and *ca.* 135 mM for inside and outside inhibition respectively. As in human red cells the asymmetry with this inhibitor was in the opposite direction from that with ethylidene glucose.

We have also studied the inhibition of exchange of 3-*O*-methyl glucose by phlorizin in cells from new-born guinea-pigs and compared this with the inhibition by phlorizin in human red cells. In both cases the inhibition of exchange was competitive but the K_1 for new-born guinea-pig cells was smaller (K_1 *ca.* 0·05 mM) than for human cells (K_i *ca.* 0·095 mM). On the other hand Cytochalasin B competitively inhibited 3-0-methyl glucose exchange with a K_1 (*ca.* 0·24 μM) which was larger than the value for human cells (0·11 μM) reported by Basketter & Widdas (1978).

Thus the hexose transfer system in new-born guinea-pig red cells has asymmetrical properties and inhibitor affinities which are similar but not identical with those in human red cells.

REFERENCES

Baker, G. F., Basketter, D. A. & Widdas, W. F. (1978). *J. Physiol.* **278**, 377–388.
Basketter, D. A. & Widdas, W. F. (1978). *J. Physiol.* **278**, 389–401.
Dawson, A. C. & Widdas, W. F. (1964). *J. Physiol.* **172**, 107–122.
Sen, A. K. & Widdas, W. F. (1962). *J. Physiol.* **160**, 392–403.
Widdas, W. F. (1955). *J. Physiol.* **127**, 318–327.

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ASYMMETRY OF HEXOSE TRANSFER SYSTEM IN ERYTHROCYTES OF FETAL AND NEW-BORN GUINEA-PIGS

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SUMMARY

1. The asymmetries of affinities of two non-transportable competitive inhibitors of hexose transfer across fetal and new-born guinea-pig erythroyctes have been studied.

2. At 16 °C 4,6-*O*-ethylidene-α-D-glucopyranose (ethylidene glucose) inhibited 3-*O*-methyl glucose exchange at 20 mM with a K_i of *ca.* 52 mM when present inside the cells and with a K_i of *ca.* 10 mM when outside. This fivefold asymmetry is qualitatively similar to but smaller than the tenfold asymmetry of human erythrocytes (Baker, Basketter & Widdas, 1978).

3. Methyl-2,3-di-*O*-methyl-α-D-glucopyranoside (trimethyl glucoside) had K_i values of *ca.* 120 mM and *ca.* 160 mM for inside and outside inhibition respectively. This is also qualitatively similar to the inhibition in human erythrocytes.

4. The inhibition produced by phlorizin, phloretin and Cytochalasin B was also studied in the erythrocytes of new-born guinea-pigs. The results were qualitatively similar to those for human erythrocytes but the inhibitory affinities were different. Thus while phlorizin and phloretin had higher affinities for the inhibition of exchange in new-born guinea-pig cells than human cells, the affinity of Cytochalasin B was less for new-born guinea-pig cells than for human cells.

5. It is concluded that the hexose transfer system in fetal and new-born guinea-pig red cells has asymmetric affinities similar to the system in human red cells but with different values of the inhibitory constants. The differences may represent species variations in a structural protein serving identical functions in the two species.

6. The possibility that fetal red cells with their facilitated transfer system play a role in sugar transport is discussed.

INTRODUCTION

Erythrocytes of fetal and new-born guinea-pigs were among those of several domestic and laboratory animals for which the fetal red cells were shown by Widdas (1955) to have a hexose transfer system similar in rate to that of erythrocytes from the human adult. The original observation by Widdas has been confirmed and extended to the rabbit by Augustin, Rohden & Hacker (1967), to the pig by Zeidler, Lee & Kim (1976) and to the dog by Lee, Auvil, Grey and Smith (1976).

Using the Sen & Widdas (1962*a*) exit technique, Dawson & Widdas (1964) studied

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the parameters of the facilitated transfer system in fetal guinea-pig red cells as a function of temperature and pH. Whereas the pH dependence of glucose transfer was similar to that in human cells, the temperature dependence was different. The maximal transfer rate was found to be larger than for human cells throughout the temperature range studied (7–37 °C) and the half-saturation constant (ϕ), although similar to that for human cells at 37 °C, did not fall in the same regular manner as the temperature was reduced. Although this different temperature dependence could indicate a difference in the membrane components involved in the hexose transfer it could have been due to a difference in the rate limiting steps in the membrane environment.

To investigate whether new-born guinea-pig cells have a similar asymmetry of affinities between inward facing sites and outward facing sites in the membrane to that of human red cells, we have studied the inhibition produced by the non-transportable inhibitors ethylidene glucose and trimethyl glucoside and also the inhibitory parameters (for equilibrium exchanges and Sen-Widdas (1962*b*) exits) of the competitive inhibitors phlorizin, phloretin and Cytochalasin B.

A preliminary report of some of these experiments was made to the Physiological Society (Aubby & Widdas, 1979).

METHODS

The majority of the experiments were done at 16 °C.

Exit experiments were carried out as described by Sen & Widdas (1962*a*, *b*). Cells were pre-incubated to contain 76 mM-glucose and exits made into 21 ml. of suspending medium by the rapid addition of *ca.* 0·15 ml. of cell suspension containing less than 3 μl. cells.

Exchange experiments were performed as described by Baker, Basketter & Widdas (1978).

Blood was obtained either from fetal guinea-pigs, near full term, as described by Dawson & Widdas (1964) or from recently new-born guinea-pigs which were stunned and bled. The blood was collected over dry heparin and rapidly mixed with phosphate buffered saline. The cells were washed three times with buffered saline before using for exchange or exit measurements.

RESULTS

3-O-methyl glucose exchange

Equilibrium exchanges can be treated as arising from a transfer system with simple kinetics in the form:

$$\text{Flux} = V_{\text{EX}} \frac{C}{C+\phi_{\text{EX}}}, \tag{1}$$

where V_{EX} is the maximal exchange flux, C is the concentration of sugar inside and outside the cells and ϕ_{EX} is the half-saturation concentration for exchange. Eq. (1) can be written in the Hanes plot form (Hanes, 1932) as:

$$\frac{C}{\text{Flux}} = \frac{1}{V_{\text{EX}}} (C+\phi_{\text{EX}}). \tag{2}$$

Thus the concentration multiplied by the reciprocal of the flux is linearly related to the concentration. In this type of plot, the intercept on the abscissa gives the half-saturation constant for exchange (ϕ_{EX}) and the intercept on the ordinate corresponds to $\phi_{\text{EX}}/V_{\text{EX}}$.

The exchange of 3-*O*-methyl glucose was measured in the range 2–160 mM using ^{14}C-labelled 3-*O*-methyl glucose and the results are shown in the Hanes plot in Fig. 1. The experiments with new-born fetal guinea-pig cells gave a half-saturation constant for exchange at 16 °C of *ca.* 25 mM which was higher than the corresponding value for human cells (*ca.* 15 mM). The value for the V_{EX} (*ca.* 167 m-mole l.$^{-1}$ min^{-1}) was also higher than for human blood (*ca.* 119 m-mole l.$^{-1}$ min^{-1}). The results for human blood are taken from Basketter & Widdas (1978).

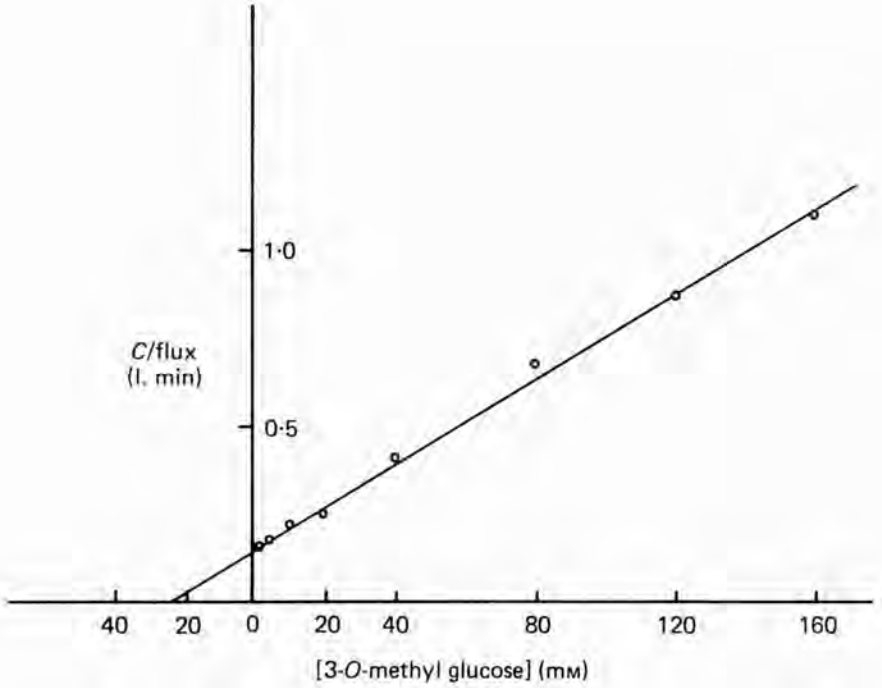

Fig. 1. Hanes plot of 3-*O*-methyl glucose exchange in the range 2–160 mM in fetal and new-born guinea-pig red cells at 16 °C. Points are the means of three experiments with similar results.

Inhibition of exchange by ethylidene glucose

When inhibitors of hexose exchange are present, a relatively simple treatment (Baker *et al.* 1978) predicts that the reciprocal of the exchange flux should be a linear function of the inhibitor concentration and that such a line should intercept a line through $1/V_{EX}$ parallel to the abscissa at a point corresponding to the half-saturation concentration for the inhibitor.

The inhibition of exchange of 20 mM-3-*O*-methyl glucose was investigated with cells equilibrated with up to 200 mM-ethylidene glucose. With the inhibitor inside the cells, osmotic compensation was provided by having inositol and malonamide in the outside medium as described by Baker & Widdas (1973). This minimizes any volume changes during the time the radioactive samples are taken to measure the exchange flux.

In Fig. 2 the reciprocal of the flux has been plotted against the inhibitor concentration for the case of internal inhibition and also for external inhibition. It can be

seen that there was marked asymmetry of inhibition. The effect of 200 mM-ethylidene glucose inside the cells was less than that of 50 mM outside. Although marked, this asymmetry was less than that obtained by Baker *et al.* (1978) in human cells. The interrupted lines in Fig. 2 represent the corresponding inhibition in human cells and it will be noted that they have similar intercepts on the ordinate. This is because at

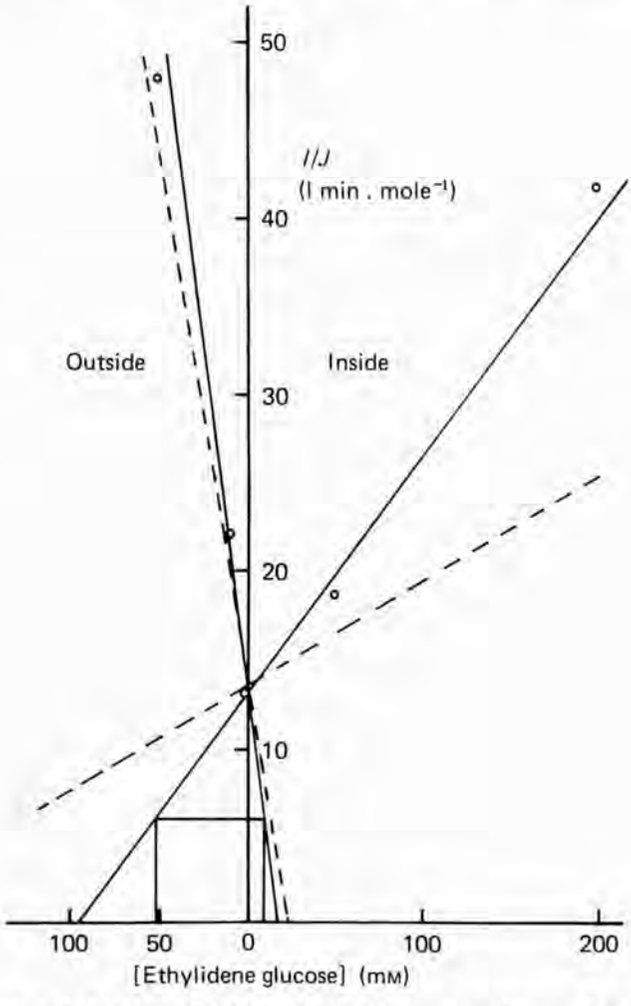

Fig. 2. Asymmetric inhibition of 3-*O*-methyl glucose exchange by purified ethylidene glucose. Points are the means of two similar results for 20 mM-3-*O*-methyl glucose exchange in new-born guinea-pig cells at 16 °C. Interrupted lines represent the corresponding results for human cells obtained by Baker *et al.* (1978).

20 mM the saturation fraction of 3-*O*-methyl glucose for human cells is greater than for new-born guinea-pig cells and this partly compensates for the lower V_{EX} in human cells.

The intersection of the lines with $1/V_{EX}$ derived from the results of Fig. 1 occurs at the K_i values for ethylidene glucose and these were *ca.* 52 mM for inhibition inside and *ca.* 10 mM for inhibition outside. The corresponding values for human red cells (Baker *et al.* 1978) were 110 mM and 11 mM. The asymmetry of the apparent affinities of ethylidene glucose was therefore fivefold in new-born guinea-pig cells in contrast to tenfold in human cells.

Inhibition of exchange by trimethyl glucoside

Methyl-2,3-di-*O*-methyl-α-D-glucopyranoside (trimethyl glucoside) was found to produce haemolysis of new-born guinea-pig cells when incubated at 200 mM to equilibrate the cells so that the inhibitor was inside. Concentrations of 100 mM inside could however be obtained with only moderate haemolysis and it was possible

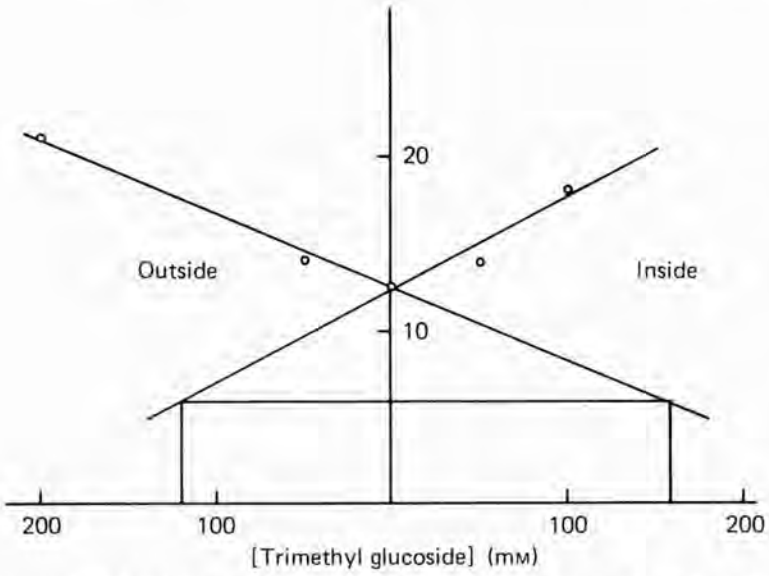

Fig. 3. Asymmetric inhibition of 3-*O*-methyl glucose exchange by trimethyl glucoside. Points are from two experiments with similar results and are for 20 mM exchange at 16 °C.

to make exchange measurements. The results are shown in Fig. 3. Although the results were rather variable, it was possible to confirm that with this reagent, inhibition was greater when the compound was inside the cells than when it was outside. That is, the asymmetry was in the opposite direction from that of ethylidene glucose. Qualitatively this was similar to the results obtained with human cells (Baker *et al.* 1978).

Inhibition by phlorizin

The inhibition of 3-*O*-methyl glucose exchange was studied at 2 and 20 mM with varying concentrations of phlorizin from 0·05 to 0·5 mM and also at varying concentrations of 3-*O*-methyl glucose from 2 to 40 mM with a constant concentration of phlorizin (0·5 mM).

A Dixon plot of the results at 2 mM and 20 mM-3-*O*-methyl glucose exchange is given in Fig. 4. The estimations of the K_i values tended to be higher at the larger concentrations of phlorizin and this suggested that the inhibitor may not have completely equilibrated with the cells at these concentrations. Experiments with phlorizin on human red cells gave similar variations of the K_i values. Calculations of the latter were made at each of the concentrations used between 0·05 and 0·5 mM and an estimate of the true K_i was obtained by extrapolation to zero phlorizin concentration.

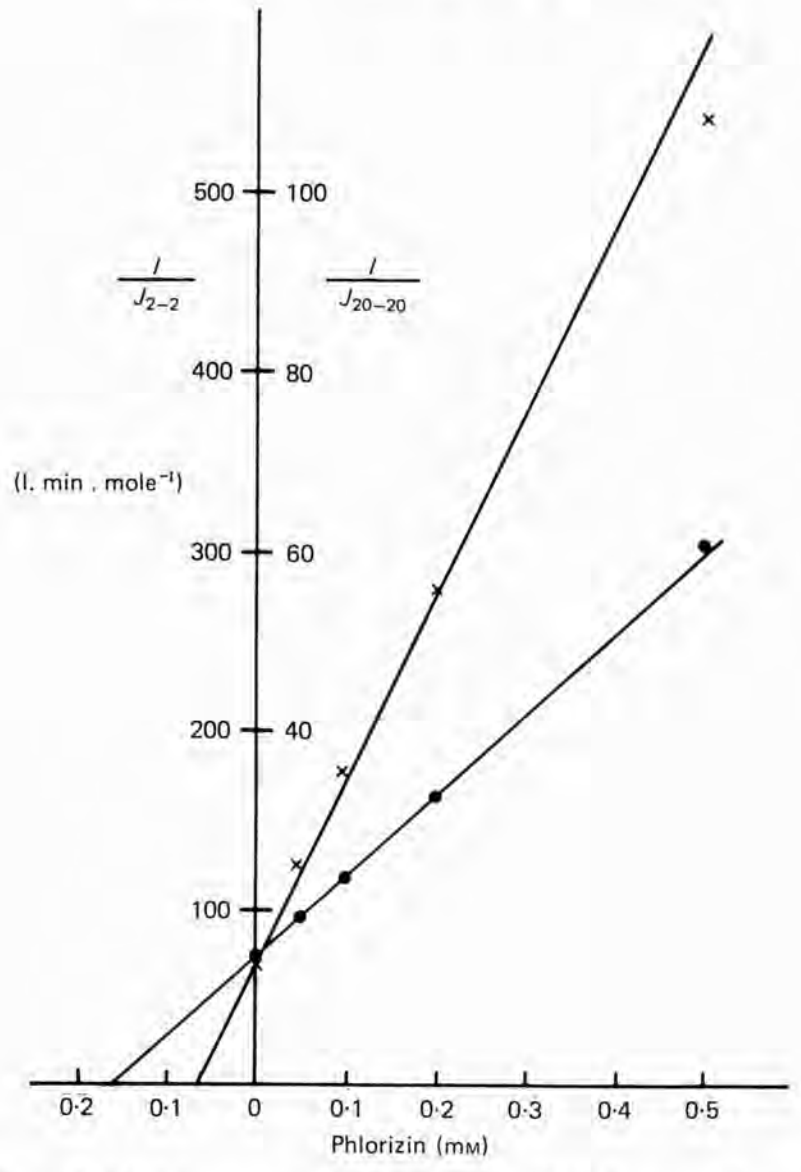

Fig. 4. Effect of phlorizin concentration on 3-*O*-methyl glucose exchange at 16 °C in new-born guinea-pig red cells. Points X, 2 mM exchange; points ●, 20 mM exchange. Points are means of two similar results at each sugar concentration.

Glucose exits were also carried out in the presence of phlorizin and the results from these and the exchange experiments are collected in Table 1.

Inhibition by phloretin

The inhibition of exchange of 3-*O*-methyl glucose by phloretin was studied in the range 0·5–2·0 μM. Sen-Widdas (1962*b*) exits of glucose were also studied in the presence of different concentrations of phloretin. Glucose exit from new-born guinea-pig cells at 16 °C was more inhibited by phloretin than from human cells and the apparent K_I for exit was only a fifth of that for inhibition of exchange.

Fig. 5 shows a typical result in which the inhibition of glucose exit in new-born guinea-pig cells is compared with that in human cells. Not only is the intercept on the

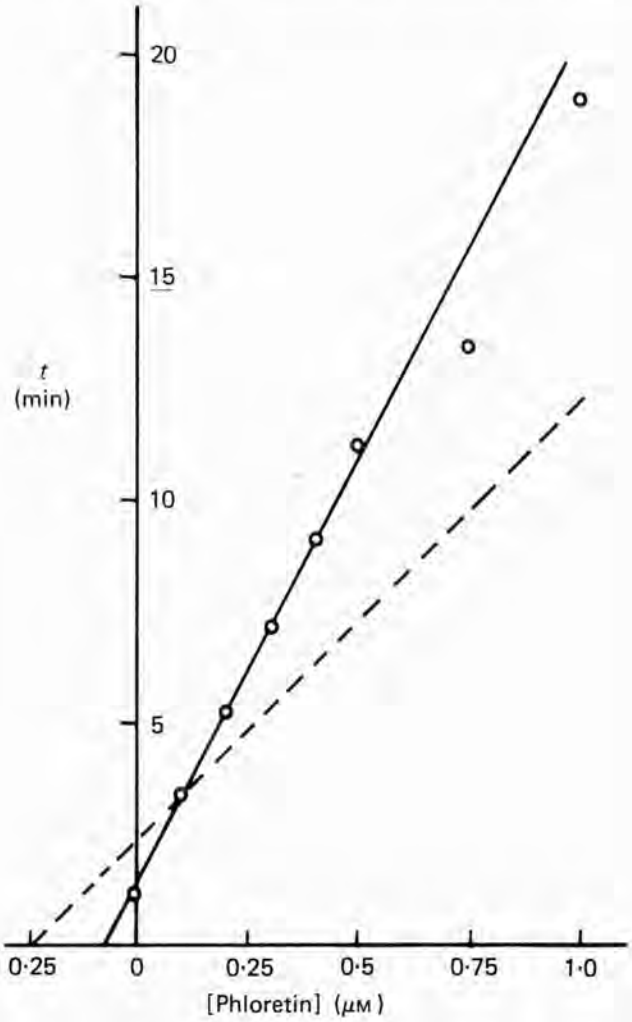

Fig. 5. Effect of phloretin concentration on glucose exit times at 16 °C. Exits were measured in a photoelectric apparatus with cells pre-incubated to 76 mM. Points and continuous line are typical results from new-born guinea-pig red cells. The interrupted line represents the corresponding results for human red cells as obtained by Basketter & Widdas (1978).

ordinate smaller, indicating a faster maximal exit rate in the absence of inhibitor, but the slope of the line is greater.

These and the results from inhibition of exchange are collected in Table I.

Inhibition by Cytochalasin B

Cytochalasin B competitively inhibited 3-*O*-methyl glucose exchange with a K_i of *ca.* 0·24 μM. The inhibition of glucose exits was also studied and the Sen-Widdas K_i was *ca.* 0·42 μM. These results are compared with corresponding results for human cells in Table 1. It will be noted that the concentration of Cytochalasin B which half-inhibited exchange was about double that for human cells but the Sen-Widdas inhibitory constant was slightly less than for human cells.

DISCUSSION

Widdas (1955) found fetal guinea-pig cells to have a faster rate of hexose transfer than human cells using glucose entry measurements, and Dawson & Widdas (1964) showed that this was true for glucose exits. The present experiments show that this also applies to hexose exchanges in fetal and new-born guinea-pig cells.

TABLE 1. Exit and exchange inhibitory constants

Inhibitor and cells	Concentration which half-inhibits glucose exit (S–W constant) μM	Inhibitory constant for exchange μM	Ratio exchange constant / S–W constant
Phlorizin			
N–b guinea-pig	50	53	1·1:1
Human	80	95	1·2:1
Phloretin			
N–b guinea-pig	0·068	0·34	5:1
Human*	0·24	0·4	1·67:1
Cytochalasin B			
N–b guinea-pig	0·42	0·24	0·57:1
Human*	0·5	0·11	0·22:1

* Data from Basketter & Widdas (1978).

This inequality does not necessarily reflect on the hexose transfer system itself since the responsible membrane components may be present in greater quantities in the fetal and new-born guinea-pig cells. The different affinities for the various inhibitors of transfer, however, more strongly suggest that there are chemical differences between the hexose transfer system of new-born guinea-pig cells and human cells, but these cannot be large since qualitatively the reactions with inhibitors are similar.

Thus the fivefold asymmetry shown towards ethylidene glucose compares with a tenfold asymmetry in human cells, but the asymmetry is in both cases a lowered affinity for the inward facing sites of the hexose transfer system. The reverse asymmetry shown by trimethyl glucoside is also seen in human cells. New-born guinea-pig cells have a greater affinity for phloretin and phlorizin than have human cells. The affinity for Cytochalasin B is less than in human cells for inhibition of exchange but is slightly greater for the inhibition of glucose exit.

The ratios of the K_i values for inhibition of exchange to those for inhibition of Sen-Widdas exits were used by Basketter & Widdas (1978) as indications of the sites of inhibition: a high value of *ca.* 6 is characteristic of outside inhibition only and a low value is characteristic of internal inhibition; inhibitors acting both inside and outside have intermediate values. It will be noted that these ratios are different for human and new-born guinea-pig cells (Table 1). In particular, the high value for phloretin may be taken to indicate that in new-born guinea-pig cells that inhibitor was acting mostly on the outside of the cell membranes whereas in human cells there was a considerable contribution from internal inhibition.

It is considered that the differences in affinities are not greater than might be

expected of a structural protein evolved in different species but serving an identical physiological function.

The physiological function of the facilitated transfer system for sugars in human red cells has never been clearly established. Since the maximal rate of transfer is at least 250 times the rate of metabolic utilization (Widdas 1954) and since the normal blood sugar would more than half-saturate the outside sites at 37 °C (Sen & Widdas

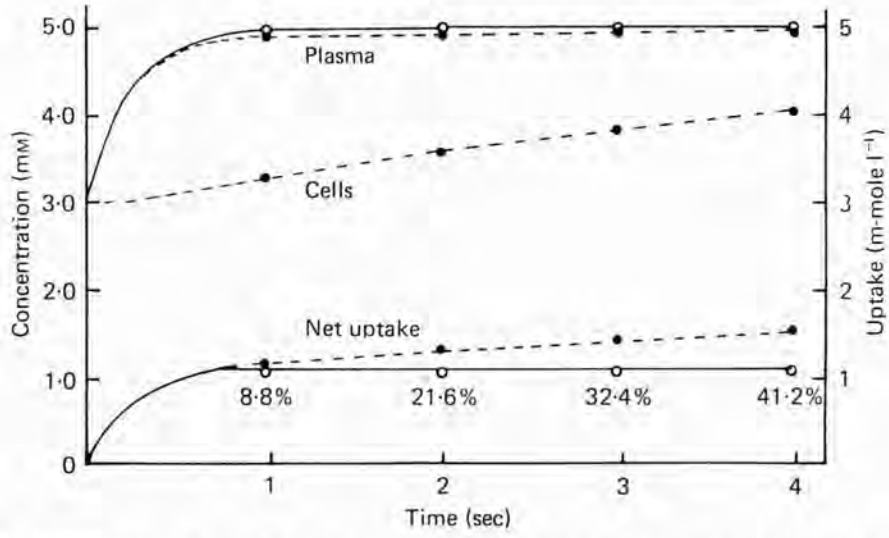

Fig. 6. Results from model calculations to show the concentration changes and uptake of glucose by blood initially equilibrated at 3 mM while in a capillary where the tissue glucose concentration was 5 mM. The uppermost lines represent the rise in plasma concentration with time in the absence (points ○ and continuous line) and presence of glucose permeable red cells (points ● and interrupted line). The middle line represents the rise in concentration in red cells whose permeability was similar to fetal guinea-pig red cells at 37 °C. The lower lines (and right hand scale) represent the net uptake of glucose if carried by plasma alone (continuous line) and if carried in both cells and plasma (interrupted line). The increment due to sugar carried in the red cells is indicated as a percentage at each sec.

1962*a*), the facilitated transfer is two orders of magnitude greater than needed for cellular metabolism. The occurrence of a facilitated transfer system with similar rates in red cells from the fetal blood of animals suggests that it may have a role in sugar transport, particularly from the placenta to the developing fetus. Thus, if transfer across the red cell membrane could occur fast enough, carriage in the red cell would give an additional capacity for transport over and above that of the fetal plasma. The cells would also have a sort of 'buffering' function in maintaining a small concentration gradient across the placenta (Widdas, 1980).

To see if this transport function would offer any practical advantage, calculations were made on a programmable calculator using the simple kinetics for asymmetric transfers described by Baker & Widdas (1973) with parameters appropriate to fetal guinea-pig cells. The calculations covered the cases of (i) uptake of glucose by blood in which the cells contributed to the uptake and (ii) the uptake of glucose by blood in which only the plasma could take up glucose. Simulations of transfer from blood to tissues were also considered.

The calculations were made at low sugar concentrations such as are likely to be met *in vivo* and it was assumed that diffusion of glucose from tissue to blood plasma would be sufficiently rapid to equilibrate in about one second, but that the equilibration between plasma and red cells would follow the facilitated transfer kinetics.

Fig. 6 shows the expected uptake by blood in a capillary bed held at a concentration of 5 mM-glucose when the blood is initially in equilibrium at 3 mM.

The result in Fig. 6 is typical of a number of similar calculations but with other starting concentrations. It shows that only a small contribution would be made by uptake in the cells during the first second although the plasma concentrations would be changing rapidly. If the blood remained in the capillary bed for 2 sec or longer, however, a significantly increased uptake would occur over and above that in a blood in which sugar carriage was in the plasma alone.

Thus blood with cells having a hexose transfer similar to that in fetal and new-born guinea-pigs would take up 22% more glucose in 2 sec, 32% more glucose in 3 sec and 41% more in 4 sec. During the first second the excess over carriage by plasma alone would only be about 9%.

The model calculations for Fig. 6 assumed a haematocrit of 45%. Carriage by the red cells could assume greater significance at higher haematocrits. Hochachka, Murphy, Liggins, Zapol, Crensy, Snider, Schneider & Quist, 1979 found the haematocrit in Weddell seal fetuses to be 70% and although the plasma glucose was always less than that of the adult the whole blood glucose was higher because of the sugar carried in the cells of fetal blood but not in the cells of the adult seal. The authors deduced a relatively slow turnover rate for glucose in that species. Rates of transfer across the red cells were not measured, but if they were as fast as in fetal guinea-pig cells the glucose uptake by blood in the placental capillaries of the seal would be incremented by more than double the percentages given in Fig. 6.

The equilibrium between cells and plasma which would be displaced due to the more rapid change in plasma concentration would be re-established (to within about 1%) in 10 sec. Thus there would be a further reduction in plasma glucose concentration before the blood reached the fetal tissues. In the tissues however, loss of glucose from the cells would help to keep up the plasma concentration.

In human and primate blood the facilitated transfer system in the cells of the adult could have a 'buffering' type action on the glucose concentration gradient on the maternal side of the placenta by the cells giving up glucose as the plasma concentration is lowered and this could add to the beneficial action of the facilitated transfer system in the cells of the fetal blood. Thus the facilitated transfer system which gives such fast rates of hexose transfer in fetal red cells of several non-primate mammals and in fetal and adult red cells of primates may be seen as serving a physiological function in the more efficient transport of glucose between mother and fetus. There is the possibility that red cells with a fast transfer of glucose were primarily evolved to serve this function during fetal life and that persistence of this property into adult life in primates is a peculiarity of primate evolution.

REFERENCES

AUBBY, D. S. & WIDDAS, W. F. (1979). Asymmetry in the hexose transfer system of erythrocytes from new-born guinea-pigs. *J. Physiol.* **293**, 73*P*.

AUGUSTIN, H. W., ROHDEN, L. F. & HACKER, M. R. (1967). Uber einige eigenschaften des monosaccharid transport systems in erythrozyten neuge borenen und envashsener kaninchen. *Acta biol. med. germ.* **19**, 723–735.

BAKER, G. F., BASKETTER, D. A. & WIDDAS, W. F. (1978). Asymmetry of the hexose transfer system in human erythrocytes. Experiments with non-transportable inhibitors. *J. Physiol.* **278**, 377–388.

BAKER, G. F. & WIDDAS, W. F. (1973). The asymmetry of the facilitated transfer system for hexoses in human red cells and the simple kinetics of a two component model. *J. Physiol.* **231**, 143–165.

BASKETTER, D. A. & WIDDAS, W. F. (1978). Asymmetry of the hexose transfer system in human erythrocytes. Comparison of the effects of Cytochalasin B, phloretin and maltose as competitive inhibitors. *J. Physiol.* **278**, 389–401.

DAWSON, A. C. & WIDDAS, W. F. (1964). Variations with temperature and pH of the parameters of glucose transfer across the erythrocyte membrane in the foetal guinea-pig. *J. Physiol.* **172**, 107–122.

HANES, C. S. (1932). Studies on plant amylases. I. The effect of starch concentration upon the velocity of hydrolysis by the amylase of germinating barley. *Biochem. J.* **26**, 1406–1421.

HOCHACHKA, P. W., MURPHY, B., LIGGINS, G. C., ZAPOL, W., CRENSY, R., SNIDER, M., SCHNEIDER, R. & QUIST, J. (1979). Unusual maternal-fetal blood glucose concentrations in Weddell seal. *Nature, Lond.* **277**, 388–389.

LEE, P., AUVIL, J., GREY, J. E. & SMITH, M. (1976). 3-*O*-methyl glucose transport in new-born and adult dog red cells. *Fedn Proc.* **35**, 780.

SEN, A. K. & WIDDAS, W. F. (1962*a*). Determination of the temperature and pH dependence of glucose transfer across the human erythrocyte membrane measured by glucose exit. *J. Physiol.* **160**, 392–403.

SEN, A. K. & WIDDAS, W. F. (1962*b*). Variations of the parameters of glucose transfer across the human erythrocyte membrane in the presence of inhibitors of transfer. *J. Physiol.* **160**, 404–416.

WIDDAS, W. F. (1954). Facilitated transfer of hexoses across the human erythrocyte membrane. *J. Physiol.* **125**, 163–180.

WIDDAS, W. F. (1955). Hexose permeability of foetal erythrocytes. *J. Physiol.* **127**, 318–327.

WIDDAS, W. F. (1980). The asymmetry of the hexose transfer system in the human red cell membrane. *Curr. Top. Membranes & Transp.* **14**, pp. 165–223. New York and London: Academic Press.

ZEIDLER, R. B., LEE, P. & KIM, H. D. (1976). Kinetics of 3-*O*-methyl glucose transport in red blood cells of new-born pigs. *J. gen. Physiol.* **67**, 67–80.